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Induction of Hepatic Lesions, Hepatomas, Pulmonary Tumors, and Hemangio-Endotheliomas in Mice With *o*-Aminoazotoluene

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The investigation reported herein was undertaken after previous experiments (*1*, *2*) had shown that the subcutaneous administration of 2-amino-5-azotoluene [1] to mice induced pulmonary tumors and also gave results suggesting that strain susceptibility to induced growths of the liver was not correlated with susceptibility to spontaneous hepatoma. The primary purpose of the experiment was to study the inheritance of susceptibility to both spontaneous and induced hepatic growths by mating mice of strains C3H and C. Strain C3H mice are susceptible to spontaneous hepatomas (*3*) and are considered resistant to induced hepatomas (*2*), whereas strain C mice are resistant to spontaneous (*4*) and are considered susceptible to induced hepatomas (*2*). If susceptibility to both types of tumors is inherited in a similar manner, the first-generation hybrids should show a correlation between susceptibility to spontaneous and induced hepatic tumors. This would be in contrast with either parent strain. If, however, susceptibility to the two tumors is inherited in a dissimilar manner, the hybrids should resemble the parent strains by exhibiting a lack of correlation in susceptibility to the two types of neoplasms.

Further hybridization was carried out by obtaining backcross animals to confirm and extend the results in the first hybrid generation. It will be seen that the investigation did not produce any conclusive results in this respect, but it is important to keep the primary purpose of the work in mind.

The experiment was also designed to study the inheritance of susceptibility to pulmonary tumors induced by subcutaneous injection of *o*-aminoazotoluene. It was known (*5*) that susceptibility to pulmonary growths produced by the administration of a hydrocarbon is inherited as a dominant characteristic and that susceptibility to the spontaneous growth parallels susceptibility to the induced tumors.

A histologic study of the changes induced in the mouse liver by *o*-aminoazotoluene is

[1] *Hereinafter referred to as o-aminoazotoluene.*

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presented since the literature does not contain an adequate description of this process. A description of the histologic structure of induced hemangio-endothelioma in the mouse is also included.

MATERIALS AND METHODS

Mice of strains C3H, C, A, and C57 Black were used. All were obtained from stocks procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and maintained at the National Cancer Institute. Strain C3H animals were used as the spontaneous-hepatic-tumor strain (*3*), since males and females more than 1 year old had shown an incidence of spontaneous hepatomas of 27 and 10 percent, respectively. Males of this strain had proved to be relatively resistant (*2*) to the induction of hepatic changes by *o*-aminoazotoluene. Strain C mice were used as the low-spontaneous-hepatic-tumor strain, since the observed incidence in this strain had been found to be less than 1 percent (*4*) and the females had been found to be very susceptible to the induced growths (*2*). These two strains fulfilled the requirements for the primary purpose of the study, inasmuch as the C3H strain is susceptible to spontaneous growths but resistant to induced changes whereas the C mice are resistant to spontaneous hepatomas but susceptible to induced changes. Moreover, it was known that hybrids obtained from these strains could be used for studies on pulmonary-tumor induction since strain C mice are considerably more susceptible to this type of tumor than are strain C3H mice (*6*).

Strain A mice were used as the high-pulmonary-tumor strain (*6*) for both spontaneous and induced growths. The C3H mice served as the low-pulmonary-tumor strain (*3*, *6*) because they are far more resistant to both spontaneous and induced tumors of the lung. It was thought that hybrids derived from strains A and C3H would furnish additional data on the inheritance of susceptibility to hepatic tumors since strain A animals are more resistant to spontaneous hepatomas than are those of strain C3H (*4*) and are believed to be more susceptible (*2*) to induced hepatic changes.

The C57 Black animals were not used in the hybridization experiment but were used in a separate experiment in which purified *o*-aminoazotoluene was employed. They are included here to show that the results obtained in the other inbred strains and their hybrids were not due to an impurity in the commercial preparation of the compound.

The experiment was started during October 1939 by mating strain A females to strain C3H males and reciprocal breeding of mice of strains C and C3H. These matings produced mice of the F_1 hybrid generation. All the inbred mice used to obtain the F_1 hybrids were marked individually and kept for 15 months or longer to ascertain the incidence of spontaneous tumors in them.

The F_1 hybrids were separated at weaning according to sex, and the mice were marked individually. Some of each litter were kept as untreated control animals, and the rest were given injections of *o*-aminoazotoluene.

Some F_1 hybrids derived from strains C and C3H were mated reciprocally to strain C mice to procure the C backcross generation, while other female F_1 hybrids were mated to strain C3H males to derive the C3H backcross generation. The F_1 males were not mated to C3H females to obtain backcross animals because the female offspring would probably develop mammary tumors before the termination of the experiment. Strain C3H is a high-mammary-tumor strain (*3*). Reciprocal breeding

was carried out between F_1 hybrids from the A×C3H cross and strain A mice. This procedure produced a small number of A-backcross-generation mice.

The sexes of all backcross litters were separated at weaning, and each mouse was marked. Part of each litter was kept to determine the occurrence of spontaneous tumors while the remainder were given injections of *o*-aminoazotoluene. This procedure gave litter-mate controls for all injected F_1 and backcross mice of the experiment. As controls for the inbred mice of strains A, C3H, and C, a comparable number of animals of the same age of each strain were given the *o*-aminoazotoluene injection.

The *o*-aminoazotoluene was obtained from the Eastman Kodak Co. and was used without purification for administration to the animals of strains C, C3H, A, and all their hybrids. As stated previously, the purified compound [2] (m. p. 102.5°–103.0°, corrected) was injected into the mice of strain C57 Black. The compound was moistened with glycerol (6 drops of glycerol to 200 mg.) and injected subcutaneously, according to technique of Shear (7), into the right axillary region. Each mouse received approximately 10 mg. each month for 10 successive months, or a total of 100 mg. They were killed 44, 48, 50, or 52 weeks after the first injection.

The mice received the first injection when approximately 2 months of age, except those F_1 hybrids used to produce the backcross generations. These hybrids were approximately 4.5 months old when given the first dose. The animals were kept under similar environmental conditions, with an unlimited supply of tap water and Purina dog chow pellets.

[2] *Obtained from Dr. J. L. Hartwell, of the National Cancer Institute.*

Necropsies were performed on all mice injected with *o*-aminoazotoluene which were killed or died after receiving the initial injection. The degree of macroscopic hepatic change was noted, and a portion of each liver was prepared for histologic studies. The untreated control mice were kept until 15 months of age or longer when they were killed and necropsied.

RESULTS

Induction of Hepatic Changes

The results of the macroscopic examinations of the livers are presented in table 1. The gross hepatic changes consisted of enlargement of the liver which was cirrhotic in appearance and often contained single or multiple hepatomas. The tumors measured from 0.2 to 1.0 cm. in diameter and bulged above the hepatic surface as globular, pink, yellowish-pink, gray, or yellow masses, covered by the smooth, intact capsule.

Microscopically, there was no extensive necrosis of parenchyma, and with the haematoxylin and eosin stain there was often very little evidence of an existing pathologic process. There was no extensive fibrosis. However, with stains for connective tissue as, for example, Laidlaw's method for demonstrating reticulum, there was a focal increase in reticulum (fig. 1, *A*). Thickened reticulum fibers were observed between the cords and sinusoids or encircling single or small groups of parenchymal cells (fig. 1, *B*). The presence of increased reticulum appeared to be correlated with the gross changes recorded in table 1. A canary-yellow pigment which was iron-negative and gave a positive acid-fast reaction with Verhoeff's stain for acid-fast bacilli was present in many Kupffer cells. In addition, some specimens showed endothelial-cell and bile-duct proliferation.

Endothelial proliferation was distributed focally, and there was considerable variation in degree. In some areas, the endothelial cells were merely prominent (fig. 2, *A*), while in others there was a numerical increase in these cells sometimes associated with loss of parenchymal cells (fig. 2, *B*). The presence of endothelial proliferation

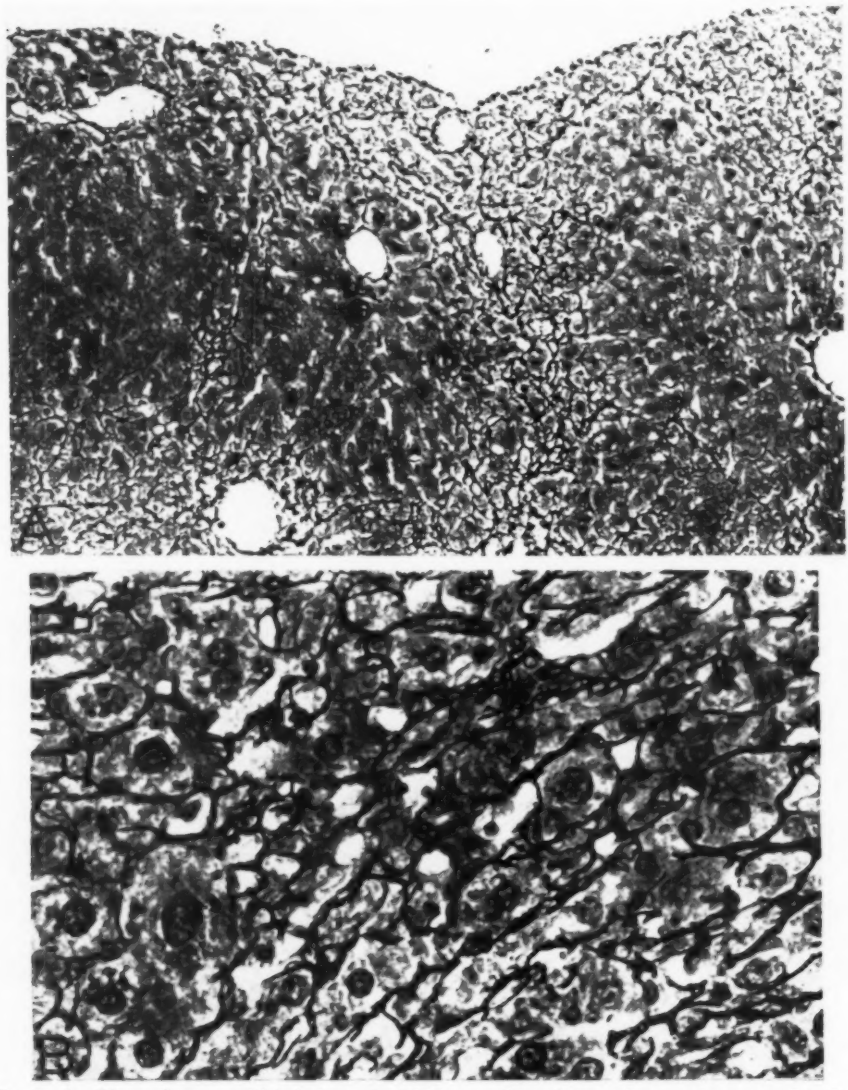

FIGURE 1.—Liver of mouse which had received 100 mg. of *o*-aminoazotoluene subcutaneously. Laidlaw's stain for reticulum. *A*, Focal increase in intralobular reticulum. × 150; *B*, Increased reticulum tending to encircle individual parenchymal cells. × 515.

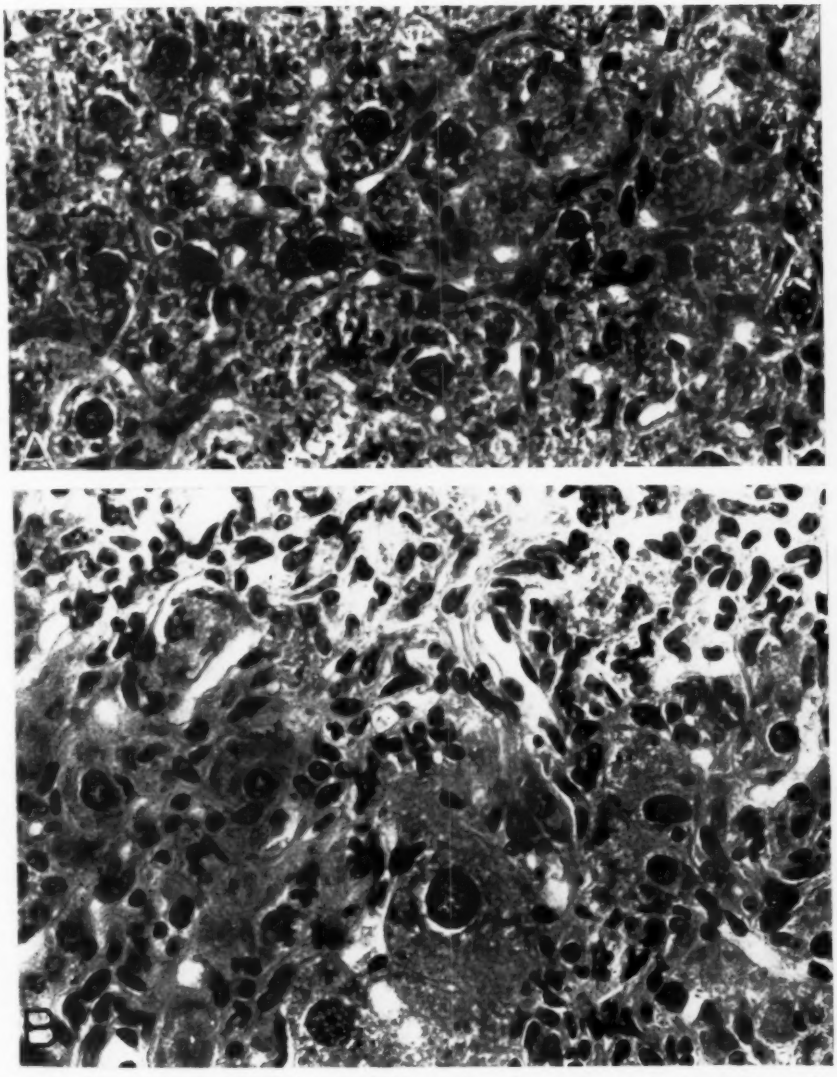

FIGURE 2.—Liver of mouse which had received 100 mg. of *o*-aminoazotoluene subcutaneously. *A*, Prominent nuclei in sinusoidal endothelial cells. × 515; *B*, Increase in endothelial cells with focal loss of parenchymal cells. × 515.

was not correlated with the macroscopic appearance of the livers. Bile-duct proliferation was not a very prominent feature, the proliferated ducts usually being restricted to the portal areas.

The tumors were all nonencapsulated and compressed the adjacent hepatic tissue, which often showed a local increase in reticulum (fig. 3, *A*). They were all well-differentiated hepatomas composed of

TABLE 1.—*The induction of macroscopic hepatic changes in mice of strains A, C3H, C, and C57 Black, and in hybrids derived from strains A, C, and C3H following subcutaneous administration of o-aminoazotoluene*

[Numerator = mice killed
Denominator = mice with hepatic changes]

Strain or hybrid generation	Derivation of hybrids	Sex	Number of mice	Mice killed or with hepatic changes (time in weeks)																Total	Incidence
				10	16	22	28	30	32	34	36	38	40	42	44	46	48	50	52		
																					Percent
A		F	11													3/3		4/4	4/4	11/11	100
A		M	11															1/0	10/5	11/5	45
C3H		M	23						1/0			8/0							14/5	23/5	22
C		F	12											1/1		2/2	1/1	4/4	4/4	12/12	100
C		M	12												1/0		3/0		8/1	12/1	8
C57 Black		F	13						1/1			1/1			11/11					13/13	100
C57 Black		M	34									1/0	1/0	2/0	30/3					34/3	9
F_1	C × C3H or C3H × C	F	33		1/0	1/0	2/0			1/0	1/1	3/2	1/1		1/1	1/1	7/6	3/3	11/11	33/26	79
F_1	C × C3H or C3H × C	M	31											1/0					30/2	31/2	6
F_1	A × C3H	F	5	1/0	1/0						1/1						2/2			5/3	60
F_1	A × C3H	M	3																3/3	3/3	100
C backcross	C × F_1 or F_1 × C	F	58						1/1	1/1	3/3				2/2	3/3	15/15	24/24	9/9	58/58	100
Do	C × F_1 or F_1 × C	M	41								1/0	2/0		3/2		1/0	2/1	22/0	10/0	41/3	7
C3H backcross	[1] F_1 × C3H	F	29											1/1		3/3	16/16	9/9		29/29	100
Do	F_1 × C3H	M	22							1/0						1/1	11/6	9/3		22/10	45
A backcross	A × F_1 or F_1 × A	F	15											5/5	1/1		4/4	5/4		15/14	93
Do	A × F_1 or F_1 × A	M	17					1/0		2/2				3/3			6/6	5/2		17/13	76

[1] F_1 = C × C3H or C3H × C.

epithelial cells arranged in liverlike cords alternating regularly with endothelial-lined blood sinuses of varying width. A strand of reticulum usually separated the sinus from the cord (fig. 3, *B*). The tumor cells had a striking resemblance to cells of the hepatic parenchyma, being cuboidal, with prominent cell margins and moderate-sized spherical vesicular nuclei, each containing one to three prominent nucleoli (fig. 4). The cytoplasm, usually faintly basophilic but occasionally eosinophilic, was finely granular or finely vacuolated. Some tumor cells showed large vacuoles. While the usual cellular picture was that of considerable regularity, uninucleate and multinucleate giant cells were observed. Mitotic figures were present in varying numbers. Nucleoli, while usually basophilic and of moderate size, were at times eosinophilic and swollen. Fat and glycogen were regularly demonstrated in abundance in the tumors examined, but the distribution tended to be focal in character. The large vacuoles were usually devoid of either of these substances, suggesting hydropic change.

Spherical cytoplasmic inclusions were observed. These were about the size of a nucleus and usually stained red with haematoxylin and eosin but were occasionally basophilic or faintly green. No pigment was observed in the tumor cells.

The tumor cords were usually one to two and occasionally five to six cells thick (fig. 5), and the sinusoids were lined with flat, simple endothelium which did not show any unusual mitotic activity. None of the tumors could be classified as adenocarcinomas. Metastases or invasion of blood vessels was not observed. Remnants of bile ducts were found irregularly in the tumors, usually at the periphery.

An interesting finding was the sex difference in susceptibility to gross hepatic changes induced by subcutaneous injection of the compound. This sex factor in susceptibility was evident in all strains and their hybrids but was more pronounced in certain groups of animals. Practically all females of each group showed hepatic changes. Five of the F_1 female hybrids derived from strains C and C3H developed mammary tumors and died before the thirty-sixth week, but of 28 survivors in the group, 26, or 93 percent, developed hepatic reactions.

The only definite evidence of different degrees of susceptibility was found in males of the various groups. Males of strain C57 Black, C, the F_1 males derived from strains C and C3H, as well as those of the C-backcross generation, showed an incidence of less than 10 percent. Strain A and strain C3H males appeared to be more susceptible than those of strain C, for 45 percent of the former and 22 percent of the latter developed hepatic changes. Eight of the C3H males died of an intercurrent infection during the thirty-eighth week, and none showed a liver change; but of the 14 survivors, 5, or 36 percent, developed the reaction. The greater susceptibility of the C3H and A males was also demonstrated in certain backcross generations; the C3H backcross males exhibited an incidence of 45 percent in contrast with the C backcross males which showed an incidence of 7 percent. The A backcross males procured by mating A $\times$ C3H hybrids to strain A males had the highest incidence (76 percent) of the males of any group.

Induction of Pulmonary Tumors

The occurrence of induced pulmonary tumors is summarized in table 2. Mice of strains C57 Black and C3H are not included in the table since none developed pulmonary tumors. The sexes are combined in the table because an analysis of the data did not reveal any definite sex

influence in susceptibility to this type of tumor.

Strain A mice were more susceptible than those of strain C, and hybrids derived from strain A were consistently more susceptible than those obtained from strain C. The F_1 hybrids produced by the C $\times$ C3H matings were of intermediate susceptibility when compared with the 54-percent incidence in strain C and 0-percent incidence in strain C3H. The strain C backcross mice revealed an inci-

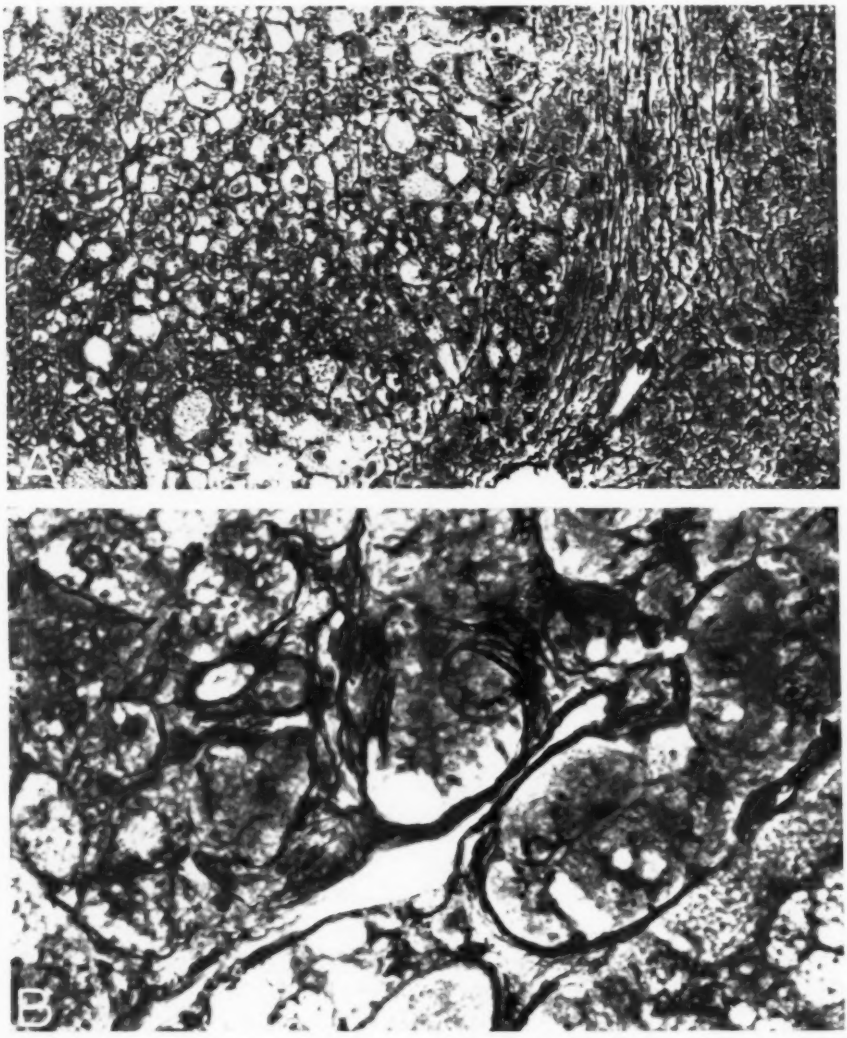

FIGURE 3.—*o*-Aminoazotoluene-induced hepatomas. Laidlaw's stain for reticulum. *A*, Rounded margin of tumor compresses adjacent hepatic tissue which shows local increase in reticulum. Note vacuolization of tumor cells. $\times$ 150; *B*, Reticulum lies between epithelial cord and sinusoid. $\times$ 515.

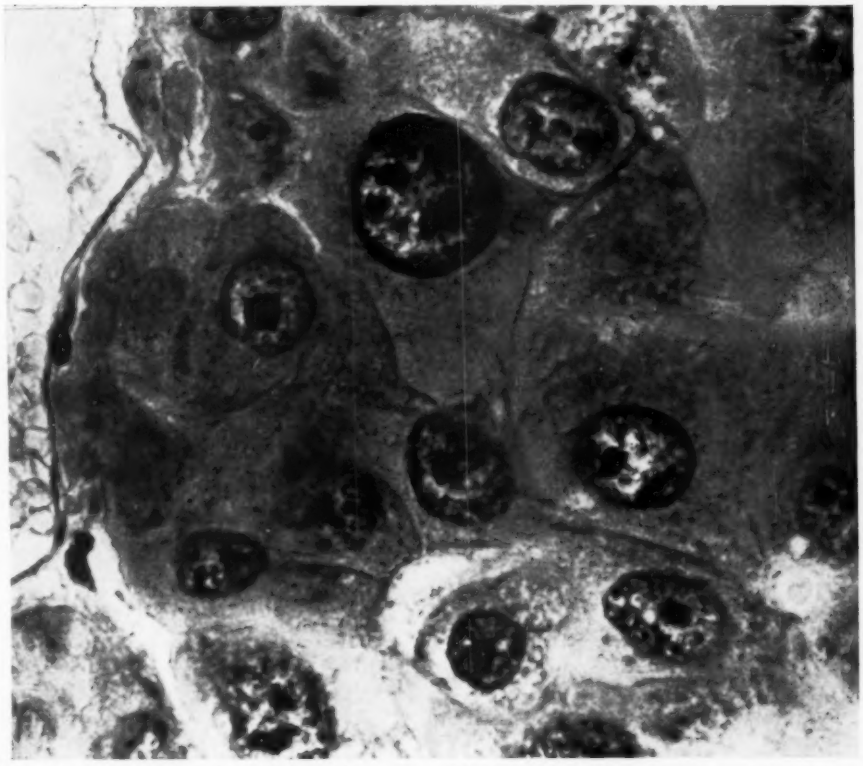

FIGURE 4.—*o*-Aminoazotoluene-induced hepatoma. Liverlike cells with prominent cytoplasmic membranes. × 1120.

dence of 46 percent whereas the C3H backcross group had an incidence of only 12 percent. It is apparent that strain C mice were more susceptible than the strain C3H animals.

Histologically, the primary tumors present in the treated mice were indistinguishable from those arising spontaneously or induced by other carcinogens (*8*).

INDUCTION OF HEMANGIO-ENDOTHELIOMA

Table 3 shows the occurrence of hemangio-endothelioma. A total of 71 hemangio-endotheliomas were found in 63 mice of the experiment. The sites of origin of the tumors according to strains and hybrid generation are summarized in table 4. Four strain C females had 2 hemangio-endotheliomas; each had a tumor in the interscapular fat, 1 had another in the peritoneal fat, 1 in the subcutaneous fat, 1 in the ovary, and 1 in the adrenal cortex. Four strain C backcross females also had 2 hemangio-endotheliomas; 2 of these had tumors in both the interscapular and peritoneal fat, 1 had tumors in the subcutaneous and intraperitoneal fat, and 1 had a tumor of the ovary as well as of the peritoneal fat. One C3H backcross female had tumors in the interscapular and in the peritoneal fat.

Grossly, the hemangio-endotheliomas were soft hemorrhagic masses ranging in

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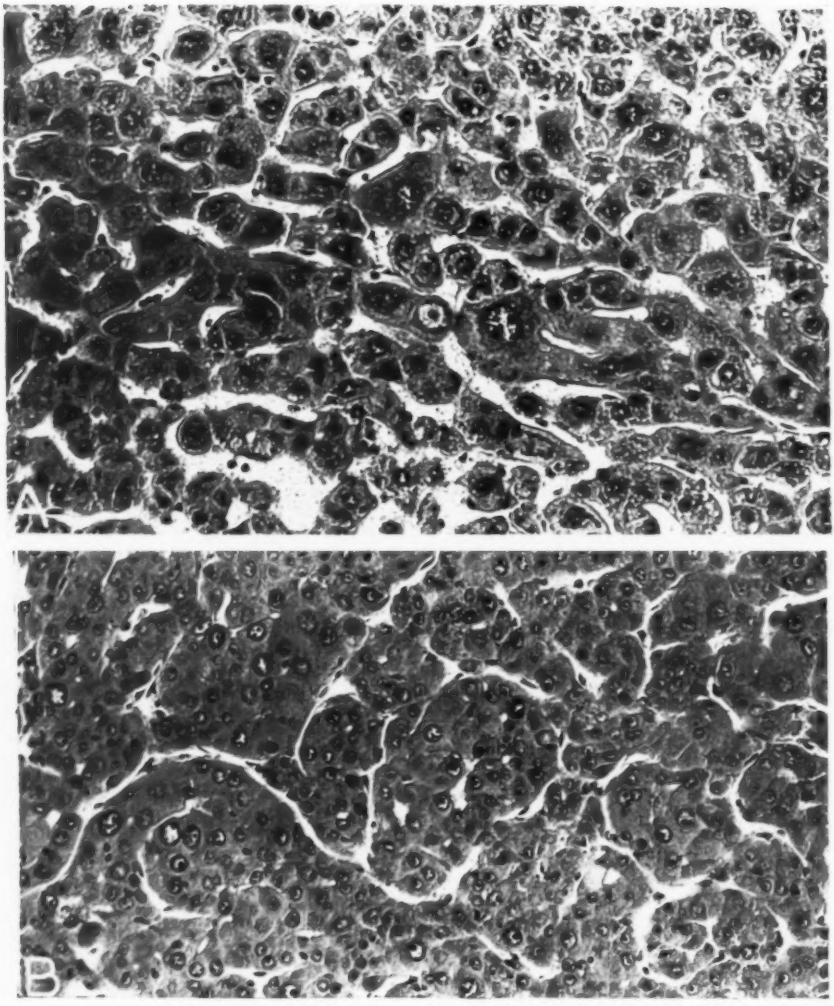

FIGURE 5.—*o*-Aminoazotoluene-induced hepatoma. *A*, Narrow cords. × 235. *B*, Wide cords of liver-like cells alternating with endothelial-lined blood sinuses. × 235.

size from 2 to 15 mm. in diameter. Most of them arose in the fat of the interscapular region (table 4) and were easily detected by palpation. Those within the subcutaneous fat depots were also noted before autopsy and were in various locations, the groin, axilla, thigh, adjacent to the salivary gland, and in the ventral cervical region. The remaining hemangio-endotheliomas were first discovered at autopsy. Those of the abdominal fat were distributed in the mesenteric fat or in the fat depots in the lower region of the abdomen; those arising within the lung, ovary, or adrenal were detectable grossly as hemorrhagic masses within the organs.

TABLE 2.—*The induction of pulmonary tumors in mice of strains A and C, and in hybrids derived from strains A, C, and C3H following subcutaneous administration of o-aminoazotoluene*

[Numerator = mice killed
Denominator = mice with pulmonary tumor]

Strain or hybrid generation	Derivation of hybrids	Number of mice	Time in weeks																Total	Incidence of pulmonary tumors
			10	16	22	28	30	32	34	36	38	40	42	44	46	48	50	52		
																				Percent
A		22													3/2		5/5	14/11	22/18	82
C		24											1/0	1/1	2/0	4/2	4/4	12/6	24/13	54
F_1	C × C3H or C3H × C	64		1/0	1/0	2/0			1/0	1/0	3/0	1/0	1/0	1/0	1/1	7/1	3/2	41/12	64/16	25
F_1	A × C3H	8	1/1	1/0						1/1						2/2		3/1	8/5	62
C backcross	C × F_1 or F_1 × C	99						1/0	1/0	4/0	2/0		3/0	2/2	4/3	17/7	46/23	19/11	99/46	46
C3H backcross	[1] F_1 × C3H	51							1/0				1/1		4/0	27/2	18/3		51/6	12
A backcross	A × F_1 or F_1 × A	32					1/0		2/1				8/5	1/1		10/7	10/9		32/23	72

[1] F_1 = C × C3H or C3H × C.

TABLE 3.—*The induction of hemangio-endotheliomas in mice of strains A, C3H, C, and C57 Black, and in hybrids derived from strains A, C, and C3H following subcutaneous administration of o-aminoazotoluene*

[Numerator = mice killed
Denominator = mice with hemangio-endothelioma]

Strain or hybrid generation	Derivation of hybrids	Sex	Number of mice	Time in weeks															Total	Incidence
				10	16	22	28	30	32	34	36	38	40	42	44	46	48	50	52		
A		F	11													3/0		4/0	4/1	11/1	*Percent* 9
A		M	11															1/0	10/1	11/1	9
C3H		M	23						1/0			8/0							14/1	23/1	4
C		F	12											1/1		2/0	1/1	4/4	4/4	12/10	83
C		M	12												1/1		3/3		8/0	12/4	33
C57 Black		F	13						1/1			1/0			11/3					13/4	34
C57 Black		M	34									1/0	1/1	2/2	30/5					34/8	24
F_1	C×C3H or C3H×C	F	33		1/0	1/0	2/0			1/0	1/0	3/0	1/0		1/1	1/0	7/0	3/0	11/0	33/1	3
F_1	C×C3H or C3H×C	M	31											1/0					30/0	31/0	0
F_1	A×C3H	F	5	1/0	1/0						1/0						2/0			5/0	0
F_1	A×C3H	M	3																3/0	3/0	0
C backcross	C×F_1 or F_1×C	F	58						1/0	1/0	3/0				2/2	3/3	15/8	24/11	9/0	58/24	41
Do	C×F_1 or F_1×C	M	41								1/0	2/0		3/0		1/1	2/0	22/1	10/0	41/2	5
C3H backcross	[1] F_1×C3H	F	29											1/0		3/2	16/4	9/0		29/6	21
Do	[1] F_1×C3H	M	22							1/0						1/0	11/0	9/0		22/0	0
A backcross	A×F_1 or F_1×A	F	15											5/0	1/0		4/0	5/0		15/0	0
Do	A×F_1 or F_1×A	M	17					1/0		2/0				3/0			6/0	5/0		17/0	0

[1] F_1 = C×C3H or C3H×C.

Microscopically, some of the hemangio-endotheliomas took the form of a purely vascular neoplasm, with capillary and cavernous blood-filled spaces lined by flat or slightly swollen endothelial cells showing varying degrees of mitotic activity (fig. 6). A more common picture, however, was one in which there was a close admixture of blood-filled vessels with more or less solid sheets of cells (fig. 7, *A*). In this type of tumor the blood vessels varied considerably in diameter and were lined either completely by simple flat endothelium, or partly by a single layer of plump cells projecting headlike into the lumen, and partly by a stratification of endothelial cells (fig. 7, *B*). The cells forming the solid sheets often resembled those lining the vascular channels, being elongated, with vesicular nuclei which contained finely divided particles of chromatin (fig. 8); while others were pleomorphic, with cuboidal, reniform, and pyriform nuclei (fig. 8). There was a tendency toward whorling of the cells (fig. 8, *A*), and very often the solid areas showed blood-filled clefts between the individual cells (fig. 8, *B*) or were closely associated with capillary (fig. 8, *A*) and wide-vessel formations. Mitotic figures and uninucleated giant cells were common. The reticulum distribution of these tumors was characterisitic of endothelioma, in a basement-membrane-like fashion about the cells forming vascular spaces (fig. 9, *A*) and concentrated about the periphery of the solid areas (fig. 9, *B*). Within the solid areas the reticulum was scanty and showed no constant relation to the individual tumor cells.

The tumors of this group lacked encapsulation and were invasive (fig. 10, *A*). Of two hemangiomas in which transplantation was attempted, the results were successful, one tumor now being in the twentieth generation.[3] The other was discontinued after the tenth generation.[4] Grossly, the transplants were often bloody cysts in the walls of which were blood-filled, endothelial-lined channels similar to the picture seen in the primary tumors (fig. 10, *B*). In addition there were papillary projections into the lumina of these cysts which were composed of sheets of large endothelial cells associated with varying amounts of connective tissue (fig. 11).

The hemangio-endotheliomas arose in all of the four inbred strains, but strain C mice appeared to be the most susceptible. It is important to note that the C57 Black mice were killed 44 weeks after their first injection, whereas most of the strain C mice were permitted to live longer. C57 Black mice may have been as susceptible as those of strain C.

The F_1 hybrids derived from strains C and C3H were much more resistant to induced hemangio-endotheliomas than were the more susceptible parent C strain. Strain C females exhibited an incidence of 83 percent, whereas the F_1 hybrid females showed an incidence of only 3 percent. When backcrossed to the parent strains, 41 percent of the C backcross females and 21 percent of the C3H backcross females developed tumors. This indicates the greater susceptibility of the strain C animals.

None of the F_1 hybrids obtained by outcrossing strains A and C3H and none of the A backcross mice developed induced hemangio-endotheliomas.

The inbred strains, as well as the hybrid generations, showed a higher incidence of the induced tumors in females than in males. This suggests a sex influence to the induction of this neoplasm.

[3] *The first transplant generation required approximately 3 weeks to become palpable.*

[4] *The first transplant generation of this tumor required approximately 4 weeks to become palpable.*

TABLE 4.—*Summary of the sites of origin of hemangio-endotheliomas induced by the subcutaneous administration of o-aminoazotoluene*

Strain or hybrid generation	Derivation of hybrids	Sex	Distribution of hemangio-endotheliomas according to site of origin						Total
			Inter-scapular fat	Perito-neal fat	Subcuta-neous fat	Lung	Ovary	Adrenal cortex	
			Number	*Number*	*Number*	*No.*	*No.*	*Number*	*Number*
A		F						1	1
A		M			1				1
C3H		M			1				1
C		F	8	1	2	1	1	1	14
C		M	2		2				4
C57 Black		F	4						4
C57 Black		M	8						8
F_1	C3H×C	F	1						1
C backcross	C×F_1 or F_1×C	F	18	5	3	1	1		28
Do	C×F_1 or F_1×C	M	2						2
C3H backcross	F_1×C3H	F	4	1	2				7
Total			47	7	11	2	2	2	71

OCCURRENCE OF TUMORS IN CONTROL MICE

The occurrence of spontaneous tumors of the liver and lung in the control animals is summarized in table 5. The inbred-strain control animals were ancestors of the hybrid generations. With the exception of strain C females, all living control mice were killed when they were 15 months old. The data in table 4 are based on these autopsy findings. Four strain A females, all the strain C3H females, 14 of the F_1 females obtained by breeding strains C and C3H, 5 F_1 females obtained by mating A females to C3H males, and 4 of the A backcross females developed spontaneous-mammary-gland tumors before they were 15 months old. Survival in the remaining mice was satisfactory. The 25 strain C females lived to an average age of 18 months before coming to autopsy.

Strain A mice and their hybrids showed the highest incidence of spontaneous pulmonary tumors. Next in degree of susceptibility were strain C mice and their hybrids.

Four strain C3H males, two F_1 males from the C × C3H cross, and one male of the C3H backcross generation developed spontaneous hepatomas. Spontaneous hemangio-endothelioma did not occur in any animal of the control series.

DISCUSSION

The experiment failed to produce any definite evidence concerning the inheritance of susceptibility to the development of spontaneous or induced hepatomas. The spontaneous growths arose only in the first hybrid generation derived by mating susceptible strain C3H to resistant strain C mice and in the C3H backcross generation, but the number of tumors was too small to permit any conclusion. The scarcity of tumors in the hybrid generations indicated that the mice were more resistant than the C3H parent strain. This greater resistance of hybrid animals was noted in other work on the inheritance of susceptibility to spontaneous mammary (9) and spontaneous pulmonary (10) tumors. The induced hepatomas occurred with approximately the same frequency in the females of all hybrid generations, and the number of this type of neoplasm in the males was too small to be of any significance.

At the beginning of the investigation it was thought that strain C animals were more susceptible than those of

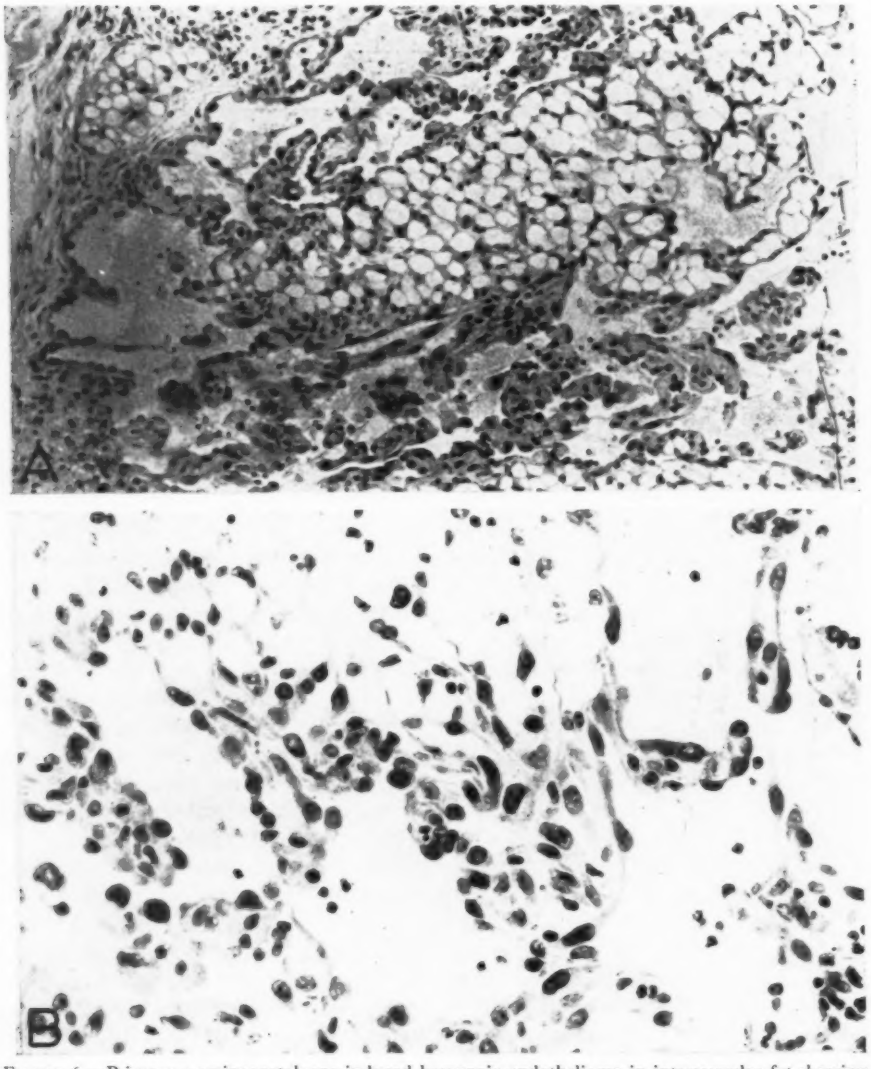

FIGURE 6.—Primary *o*-aminoazotoluene-induced hemangio-endothelioma in interscapular fat showing a purely vascular structure. *A*, × 145; *B*, × 380. See sixth-generation transplant in figure 11, *A*.

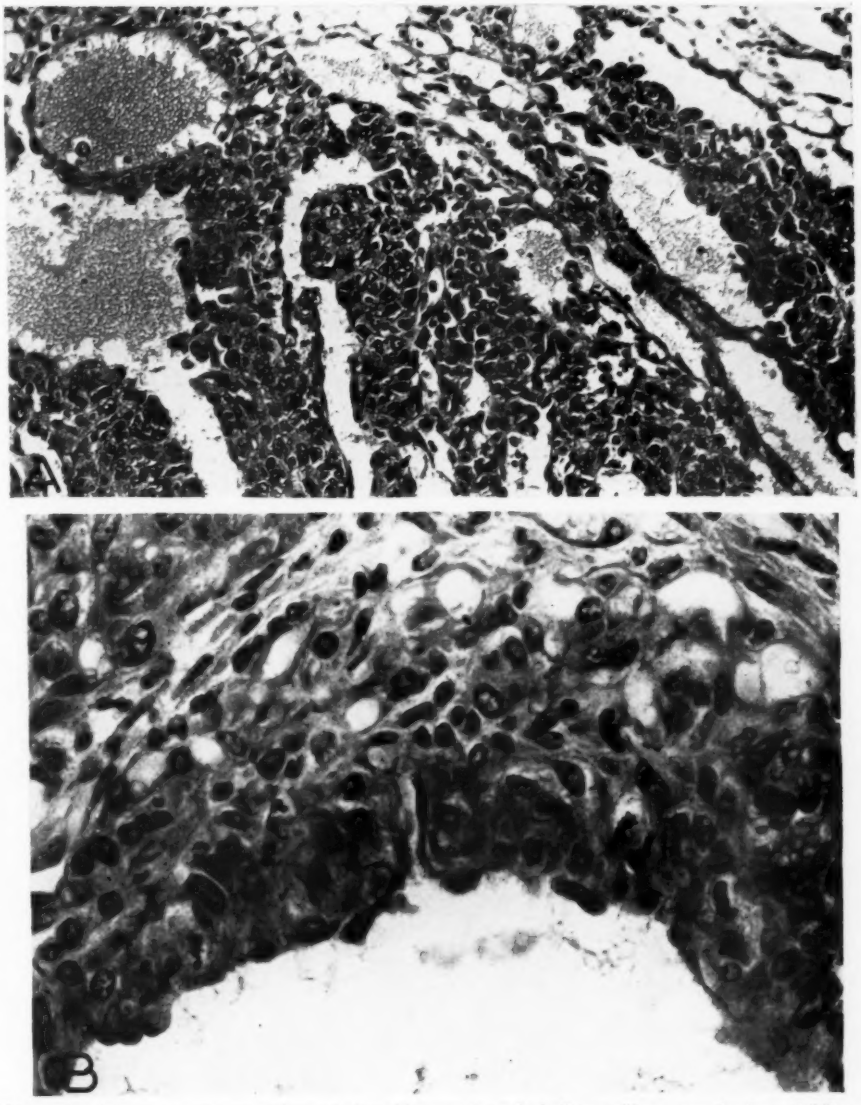

FIGURE 7.—Primary *o*-aminoazotoluene-induced hemangio-endothelioma of interscapular fat. *A*, Mixture of vascular and solid areas. × 185; *B*, Large tumor-blood-vessel wall formed by stratification of endothelial cells. × 515.

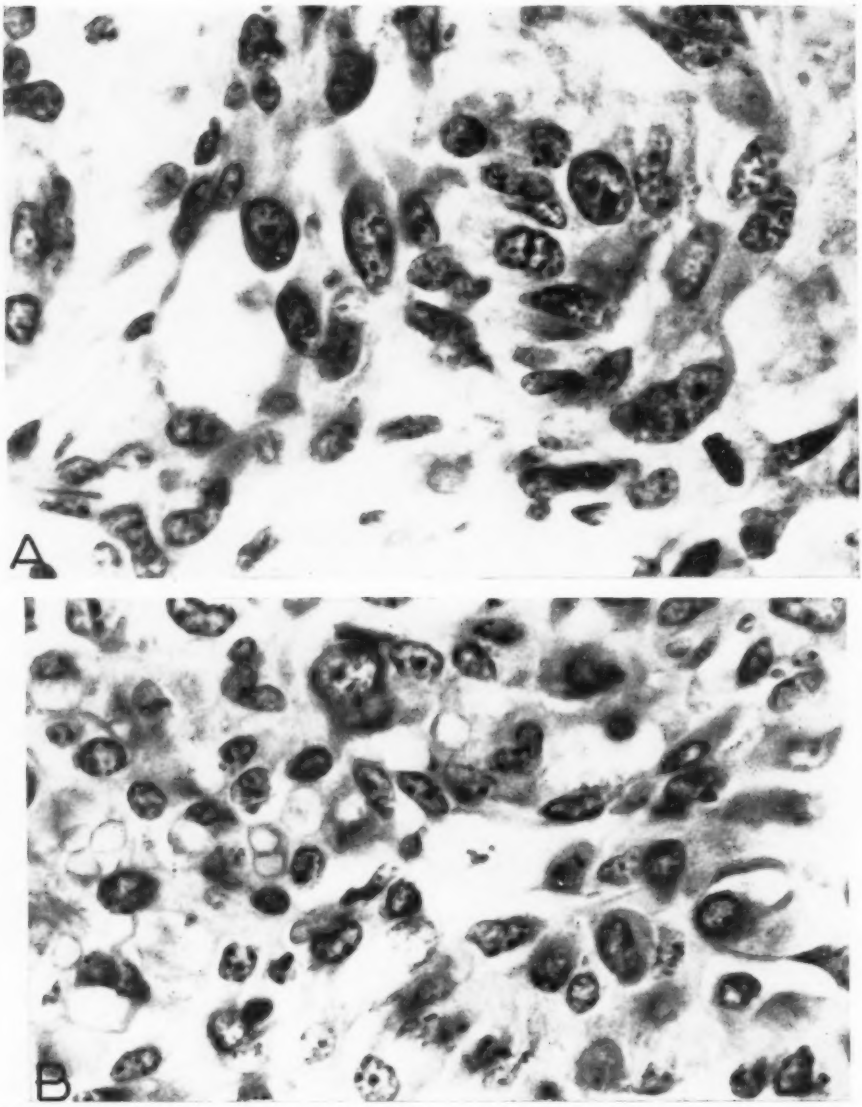

FIGURE 8.—Solid areas in primary *o*-aminoazotoluene-induced hemangio-endothelioma. *A*, Tendency for concentric arrangement of cells. One tumor capillary present. × 1050; *B*, Red-blood-cell-containing clefts among tumor cells. × 1050.

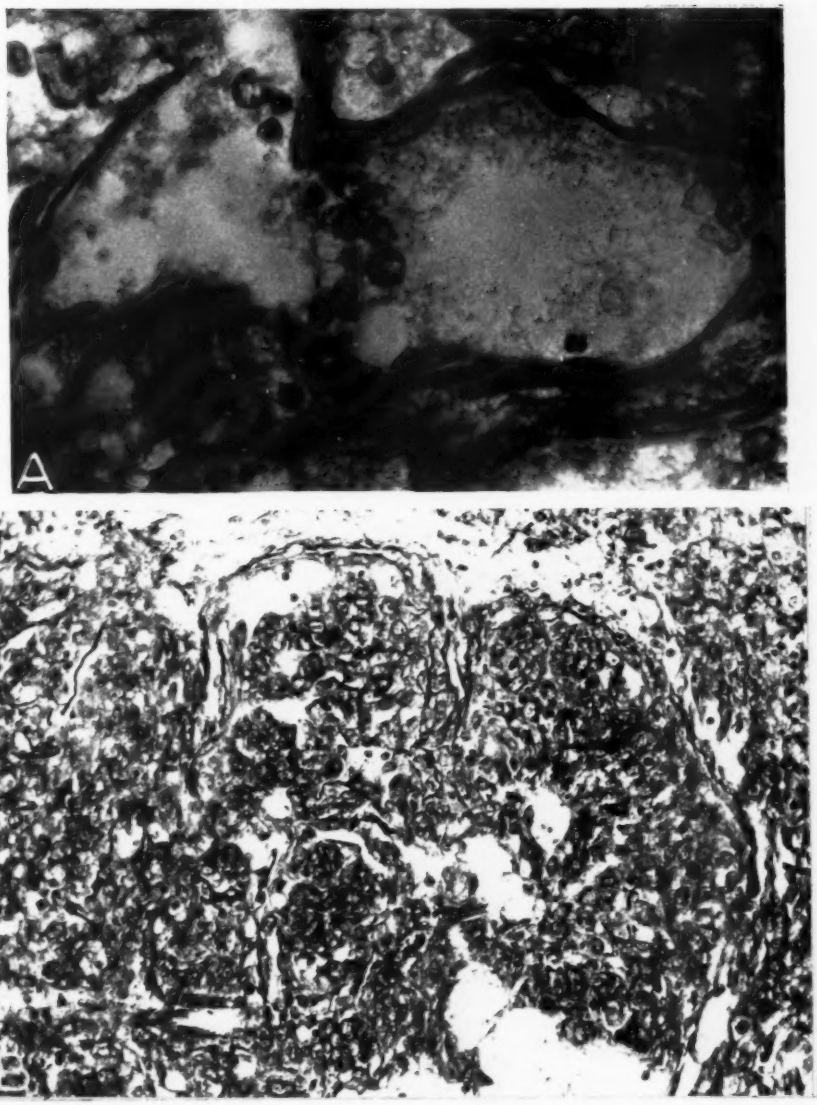

FIGURE 9.—Reticulum distribution in *o*-aminoazotoluene-induced hemangio-endothelioma. Laidlaw's stain for reticulum. *A*, Basement-membranelike distribution about a capillary. × 860; *B*, Concentration about periphery of solid areas. × 260.

strain C3H to the induction of hepatomas with *o*–aminoazotoluene since an earlier experiment (*2*) showed that strain C females were considerably more susceptible than strain C3H females. In view of the pronounced sex difference in susceptibility to hepatic reactions induced by the compound, as shown here, this assumption was not justified. The incidences of hepatic reaction in the males of strains C and C3H in the present work suggest that the C3H mice may be the more susceptible. Considerable work is necessary before the strains of mice can be arranged in order of their susceptibility to induced hepatomas.

The sex factor in susceptibility to induced hepatic changes was noted and discussed in a recent communication (*11*), which reported the same phenomenon following subcutaneous administration of 3, 4, 5, 6–dibenzcarbazole or *o*-aminoazotoluene to mice. The castration of male mice prior to the injection of 3, 4, 5, 6–dibenzcarbazole increased their susceptibility, but stilbestrol did not increase the susceptibility of castrated males. On the basis of evidence presented by Wicks (*12*), it was suggested that the males may be able to excrete more of the carcinogens. The problem is receiving further consideration.

In this investigation, it was found that in general those livers showing gross reactions contained an increase in reticulum and endothelial proliferation, whereas a considerable number of normal-appearing livers showed endothelial proliferations only. The microscopic studies suggested, therefore, that the gross changes were associated with an increase in reticulum and not with the presence of endothelial proliferation.

As already indicated, the structure of the *o*-aminoazotoluene-induced tumors of the liver in the mice varied to some degree. However, in all the tumor cell was sufficiently similar to the hepatic parenchymal cell to allow easy recognition of the relationship between the two. In the material reported by Shear (*7*), this feature was likewise evident, although obvious atypicality as evidenced by giant cells and wide cords of tumor cells appears to have been more common. It is of interest that in the mouse the *o*-aminoazotoluene-induced hepatomas are essentially similar structurally to carbon tetrachloride-induced (*4*) and spontaneous hepatomas (*13, 14*). All these tumors have in common the ability to store fat and glycogen, and the Golgi apparatus is similar in all (*15*). The two varieties of induced hepatomas exhibit an identical mitochondrial pattern, which, however, differs from that in the spontaneous variety of hepatoma (*15*).

The induction of pulmonary tumors in mice of strains A and C by subcutaneous injections of *o*-aminoazotoluene confirmed earlier observations (*1*). The present work also showed that susceptibility to pulmonary growths produced by the compound is inherited in a dominant manner and that susceptibility to spontaneous tumors of the lungs paralleled susceptibility to the induced growths. In this respect, the response of the lungs of mice to *o*-aminoazotoluene injections was similar to that produced by hydrocarbons (*9, 16*). In addition, the pulmonary tumors induced by the compound were indistinguishable from those produced by other carcinogens (*7, 17*).

Further evidence of the ability of the compound to induce tumors in tissues other than the liver was the occurrence of hemangio-endotheliomas in the *o*-aminoazotoluene-treated mice. This tumor occurred in all the inbred strains and in certain groups of hybrids, but mice of strain C appeared to be the most susceptible. Most of the tumors were within fat

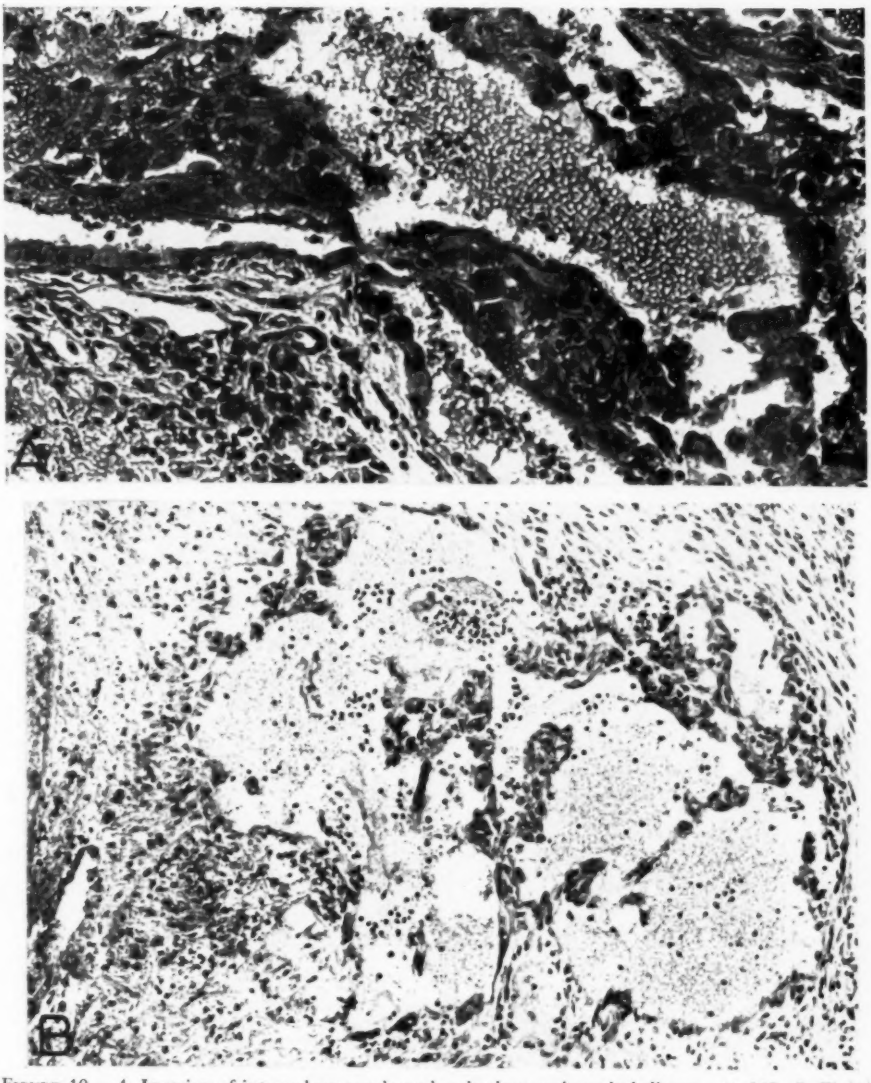

FIGURE 10.—*A*, Invasion of intrapulmonary bronchus by hemangio-endothelioma. × 265; *B*, Eighth-generation transplant of *o*-aminoazotoluene-induced hemangio-endothelioma. Wide vascular spaces. × 145. See sixth generation of this tumor in figure 11, *B*.

depots in the interscapular region, abdomen, or subcutaneous tissues, and none occurred at the site of injection. The reason for this predilection for fatty tissue is obscure.

It is believed that there are no previous reports claiming *o*-aminoazotoluene as an agent capable of inducing hemangio-endothelioma, although other investigators have reported the induction of this

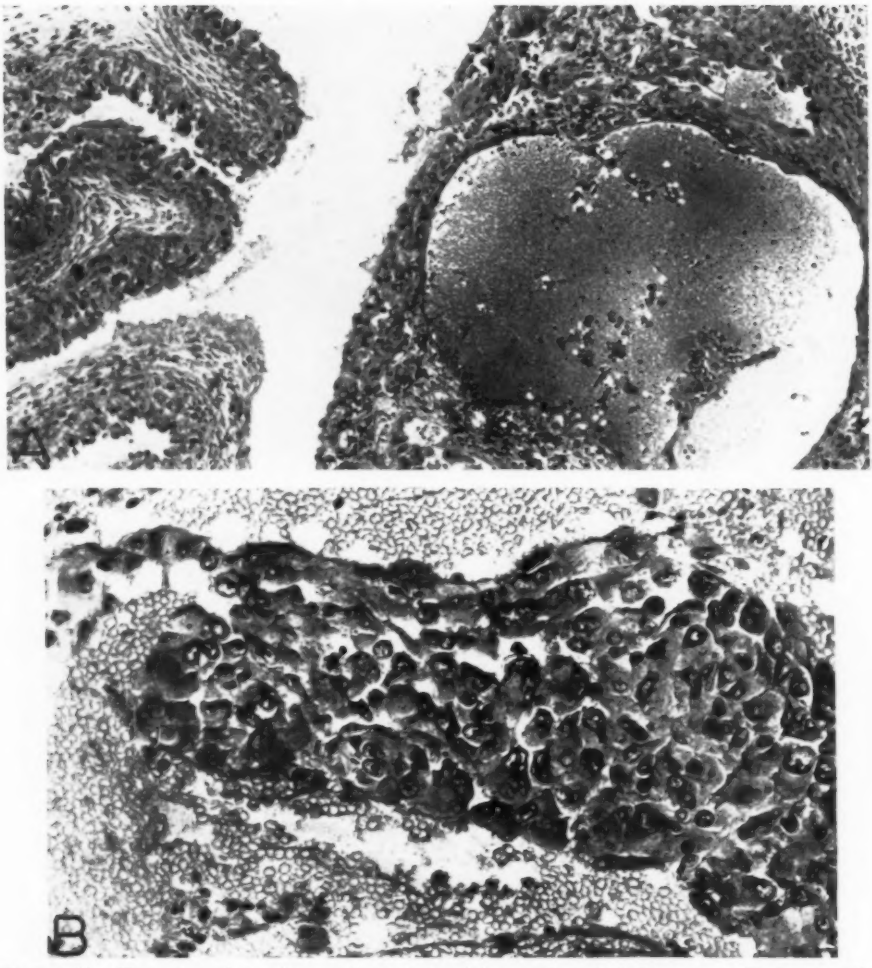

FIGURE 11.—Transplanted *o*-aminoazotoluene-induced hemangio-endotheliomas. *A*, Sixth generation of the primary tumor shown in figure 6. Vascular spaces and connective tissue papillae covered by endothelial cells. × 100; *B*, Sixth generation of same primary tumor as in figure 10, *B*. Solid sheets of endothelial cells. Eighth generation of this tumor is shown in figure 10, *B*. ×350.

type of tumor at or near the site of administration of colloidal thorium dioxide (*18*, *19*), polycyclic hydrocarbons (*20–23*), and exposure to ultraviolet light (*24*). The occurrence of the tumor at or near the site of administration in the investigations referred to above is in contrast with the present findings. It is clear that *o*-aminoazotoluene need not be injected directly into a tissue to induce hemangio-endotheliomas at the site. Following subcutaneous injection, tumors were obtained in the lung, adrenal, ovary, and abdominal fat. Even the subcutaneous tumors, most of which developed in the interscapular fat, were some distance from the injection

site. Law (*25*) reported the induction of fibrosarcomas at the site of subcutaneous injection of olive-oil solutions of *o*-aminoazotoluene into C57 Black mice. Turner and Mulliken (*26*) induced sarcomas in the interscapular region of strain C mice by the subcutaneous injection of a corn-oil solution of the compound. These latter authors did not classify the tumors as to cell type, and their report contained no illustrations. It is evident that the tumor-inducing activity of the compound is not limited to the liver.

The hemangio-endotheliomas were composed of atypical blood-vessel endothelium which formed both vascular channels and solid areas. As a general rule when a primary tumor yields metastases or is subjected to transplantation, the secondary neoplasms closely resemble the primary tumor microscopically. A notable exception, however, may be found in the case of hemangio-endothelioma. We have collected a number of metastasizing hemangio-endotheliomas in human beings. In one group both the primary neoplasm and the metastases appeared malignant histologically. In the remainder, although the primary tumor had a benign structure, the metastases appeared either benign or malignant (*27*). A spontaneous hemangio-endothelioma of the mouse liver studied in this laboratory revealed that the primary tumor was highly differentiated and almost purely angiomatous. Subcutaneous transplants showed both solid and vascular areas, while the metastases from the transplants had the suggestively benign histologic appearance of the primary tumor (*27*).

In view of this experience with hemangio-endothelioma, we hesitate to classify the tumors reported herein as benign or malignant on the basis of histologic examination alone. Even in the case of the two tumors in which the transplantation was carried out successfully, the question remains open, for in none of the mice carrying transplants was there any evidence of metastases. According to our present knowledge, transplantation alone should not be considered as an absolute indication of malignancy.

In the case of the mice with multiple hemangio-endotheliomas, two possibilities exist—that we are dealing with primary tumors of multi-centric origin or with primary and metastatic lesions. This awaits further clarification.

Spontaneous hemangio-endothelioma in the mouse may arise at different sites

TABLE 5.—*Occurrence of spontaneous tumors in control mice*

Strain or hybrid generation	Derivation of hybrids	Sex	Number of mice	Mice living to 15 months of age	Mice with pulmonary tumors	Incidence of pulmonary tumors	Mice with hepatic tumors	Incidence of hepatic tumors
				Number	*Number*	*Percent*	*Number*	*Percent*
A		F	12	8	5	62	0	
A		M	2	2	1	50	0	
C3H		F	7	0				
C3H		M	10	10	0	0	4	40
C		F	25	25	4	16	0	
C		M	5	4	1	25	0	
F_1	C×C3H or C3H×C	F	32	18	1	5	0	
F_1	C×C3H or C3H×C	M	31	30	2	6	2	7
F_1	A×C3H [1]	F	7	2	0	0	0	
F_1	A×C3H	M	4	3	2	67	0	
C backcross	C×F_1 or F_1×C	F	54	54	4	7	0	
Do	C×F_1 or F_1×C	M	40	38	5	13	0	
C3H backcross	[1]F_1×C3H	F	28	28	0	0	0	
Do	[1]F_1×C3H	M	24	20	0	0	1	5
A backcross	A×F_1 or F_1×A	F	14	10	5	50	0	
Do	A×F_1 or F_1×A	M	19	14	3	21	0	

[1] F_1=C×C3H or C3H×C.

including the subcutaneous tissue, spleen, liver, and ovary (*27, 28, 29*). However, the absence of this tumor in the litter-mate controls of the injected mice in this experiment permits the conclusion that the hemangio-endotheliomas were induced. Furthermore, the occurrence of the tumor in the C57 Black mice injected with a highly purified lot of the compound rules out the possibility that the tumors were induced by an impurity in the commercial preparation.

SUMMARY

Subcutaneous injections of *o*-aminoazotoluene moistened with glycerol were given to mice of strains A, C3H, C, C57 Black, and hybrids derived from strains A, C3H, and C.

The compound induced hepatic lesions and hepatomas in all inbred strains and in the hybrids. The females of all groups were far more susceptible to induced macroscopic hepatic lesions and hepatomas than were the males.

The compound also induced pulmonary tumors in mice of strains A and C and their hybrids. These tumors were indistinguishable microscopically from those arising spontaneously or induced by other carcinogens. The inheritance of susceptibility to the *o*-aminoazotoluene-induced pulmonary tumors was similar to that of spontaneous or hydrocarbon-induced pulmonary growths.

Hemangio-endotheliomas were induced in all inbred strains and in certain hybrid groups. These tumors involved a variety of sites remote from the site of administration.

The compound when moistened with glycerol is capable of eliciting tumors in tissues distant from the site of subcutaneous injection, and its carcinogenicity is not restricted to hepatic tissues.

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Production of Subcutaneous Sarcomas in Mice With Tars Extracted From Atmospheric Dusts

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During the last decade, primary carcinoma of the lung has come to be recognized as one of the common forms of cancer in man, and considerable attention has been devoted to the question of possible etiologic factors (*1–3*). Experimentally, Passey (*4, 5*), Campbell (*6–10*), and others have shown that certain materials, which are common sources of air pollution, are capable of producing neoplasms in mice.

The collection of dusts present in the atmosphere and their examination for carcinogenic potency in experimental animals was begun in these laboratories in 1936. In the first study, carried out in collaboration with J. Siegel, Director of the Chemical Laboratory of the Air Pollution Survey of New York City, negative results were obtained with tars extracted by organic solvents from sedimented city air dust.[1]

Accordingly, other collection and extraction techniques were employed in the present study. Dusts were obtained from several cities as follows: by electrostatic precipitation and by filtration through cotton cloth, in Chelsea, Mass., and in Pittsburgh; by labyrinth precipitation from the intake and exhaust air in the Holland Tunnel connecting New York City and Jersey City; and by filtration through cotton cloth in Cambridge, Mass. The collection sites were chosen so as to obtain representative samples of dust inhaled by the general population, and not by a group of industrial workers exposed to a selected occupational condition.

The tarry matter was extracted from the dust specimens and dispersed in a fluid vehicle. About 50 mg. of tar was given in a single subcutaneous injection to mice of inbred strains. Sarcomas began to appear at the injection site after 5 months.

For clarifying the situation as regards man, it is, of course, necessary to study

[1] SIEGEL, J., *and* SHEAR, M. J.: *Unpublished work. (1938).*

Air dust was collected by the "soot-fall" method at 150 locations in all 5 boroughs of New York City (11). *Some were high locations, i. e., at a height of 20 to 86 stories; the others were the roofs of low buildings. At first, the tarry matter was extracted with carbon disulfide; later, the samples were extracted with carbon tetrachloride. The total amount of tar obtained from the specimens collected at all these locations was about 0.3 gm. per month.*

The tars were dispersed in the fraction of lard fluid at 38° C., as obtained by filtration through paper, and injected subcutaneously in 40 strain A mice of both sexes. The tar specimens from Manhattan were combined and given to 1 group of 20 mice; the tar specimens from the 4 other boroughs were combined and given to another group of 20 mice. The first injection (Feb. 3, 1937) consisted of 0.25 cc. of preparations containing about 15 mg. tar per cubic centimeter of lard filtrate. In subsequent injections the concentration of tar was progressively increased to 35 mg. per cubic centimeter and the volume increased to 0.5 cc. The total amount of tar injected was about 50 mg. per mouse. About half of this amount was given in 5 divided doses during the first 6 months; the other half was given in 2 injections during the next 5 months.

Survival was good. Of the 40 mice, 37 were alive at 11 months when the last injection was given. At 15 months 27 were alive. The experiment was terminated in the seventeenth month at which time 22 of the mice were still alive.

Ulceration at the site of injection was noted in only four mice. No tumors were obtained in any of the mice at the injection site, and no noteworthy increase was observed in the number or size of pulmonary tumors, which occur spontaneously in mice of this strain.

the problem clinically and epidemiologically. The experiments reported in this paper were restricted to the mouse, and our interpretations of them are, for the present at least, also restricted to this experimental animal. Even in the case of the mouse, however, these experiments provide no information regarding the production of pulmonary tumors by inhalation of the dusts; they deal only with the results of injection of dusts and of tars extracted from them.

METHODS AND APPARATUS

Apparatus and Location

The mechanical filter employed in Chelsea and in Pittsburgh consisted of an electric motor-driven blower, with an inlet duct extending through a window to the outside atmosphere and with an exhaust duct in the shape of a Y. A filter bag was attached to each of the two ends of the exhaust duct. The bags were made of a dense felted cotton fabric similar to that used for vacuum cleaner bags. The capacity of the filter was about 200 cubic feet of air per minute.

The electrical dust collector was a Westinghouse Precipitron, Room Unit, Model R-39, with a capacity of 240 cubic feet per minute. From an inlet duct through a window, the street air was drawn through the collector cell by an electric motor-driven blower. The collector cell consisted of two essential parts, viz, an ionizing chamber and a precipitating chamber. In the ionizing chamber an electric charge was conferred upon the suspended dust particles. On passing into the precipitating chamber, these charged particles were discharged on plates bearing an opposite charge, to which they then adhered.

In the Holland Tunnel the intake air was drawn from the outside through a labyrinth consisting of a series of baffles which offered opposition to the directional motion of the air and caused deposition of suspended particles by impingement. The exhaust air from the tunnel was passed through a similar labyrinth before being discharged to the outside.

In the Converse and Mallinckrodt Chemical Laboratories of Harvard, in Cambridge, the dust was collected in the ventilating system of the buildings. In this instance the intake air was drawn through a large number of untreated cotton bags which retained the suspended dust.

In Chelsea, the filtering apparatus and the precipitron were installed in a room on the second floor of a building in the Marine Hospital. Although the hospital buildings stood on a large grassy tract of land there were, nearby, many large industrial plants and much shipping at the wharves. In Pittsburgh, the filtering apparatus and the precipitron were installed in a room on the second floor of the Post Office Building located at 7th and Grant Avenues in the downtown business district, about 1 block from a large railroad station. In Cambridge, the laboratory buildings were in a residential area: the filters of the ventilating system were in the attic. In the Holland Tunnel, the intake air was drawn into ventilating towers located above the tunnel about one-half mile from the vehicular entrances at both ends.

Collection and Extraction of Dusts

All the collectors operated continuously. A vacuum cleaner was used to remove the dust from the cotton filter bags in Cambridge; the dust was obtained from the vacuum cleaner by shaking. At the Holland Tunnel, the dust collected in the labyrinths is removed by the truckload

at intervals of about 2 years. Specimens [2] were taken at random from the large amount of material in the labyrinths and passed through a 40-mesh sieve to remove the large particles. The dust in the precipitron, in Chelsea and in Pittsburgh, was removed by dismantling the collector cell and plates and brushing off the collected material with a stiff brush. In the filter bags in Chelsea and in Pittsburgh the dust was for the most part imbedded in the nap of the fabric and could not be readily removed. The bags and their contents were extracted without prior removal of the dust; the bags had been similarly extracted before use to remove from the fabric such constituents as were soluble in the solvents employed.

The above-described materials were extracted with reagent-grade benzene and then with reagent-grade ethyl ether in large Soxhlet extractors until the extracts were colorless. This required continuous treatment for 24 to 48 hours with 5 to 10 extractions per hour. After filtration through paper to remove suspended matter, the extracts were freed from solvent at temperatures not exceeding that of the steam bath.

The materials extracted from each specimen by benzene and by ether were combined and dispersed in a sample of synthetic tricaprylin (m. p. 6°–7° C.) which was prepared by Dr. J. L. Hartwell (*12*). Filtered lard was used as the vehicle in the case of the tar from Cambridge. Since some of the tarry material remained undissolved, the preparations were shaken before injection. Data on the extracts are given in table 1.

[2] *Obtained through the cooperation of Dr. Edward Levy, medical director, and John A. Lee, superintendent, both of the Port of New York Authority.*

Biologic Tests

Male mice of the C3H strain and mice of both sexes of the A strain were used for the biologic testing of the extracts. The animals, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were 2 to 3 months old at the start of the experiment in July 1940. They were offered unlimited amounts of Purina dog chow and tap water. The females were kept separate from the males.

Unextracted Dusts

Six specimens of unextracted dust were injected subcutaneously into C3H male mice. The dusts were suspended in a 0.9-percent solution of sodium chloride to which a small amount of the dioctyl ester of sodium sulfosuccinate (Aerosol O. T.) was added as emulsifying agent (*13*). A single injection of 0.25 cc., containing 20 mg. of dust, was given in the right axilla. Twenty mice were employed for each specimen.

After diluting these suspensions 1:8,

Table 1.—*Tarry matter extracted from atmospheric dusts*

Location	Collection method	Weight of dust	Benzene extract		Ether extract	
		Grams	*Grams*	*Percent*	*Grams*	*Percent*
Chelsea	Filter bag	[1]	7.5			
Do	Electric precipitron	20	.8	4.0	0.04	0.20
Pittsburgh	do	100	9.4	9.4	.06	.06
Do	Filter bag	[1]	10.9			
Holland Tunnel:						
New York intake	Labyrinth	30	.8	2.7	.01	.03
New York exhaust	do	50	5.3	10.6	.01	.02
New Jersey intake	do	88	4.5	5.1	.03	.03
New Jersey exhaust	do	75	9.2	12.3	.26	.35

[1] Weight of dust not obtainable with accuracy. (See above.)

they were administered intravenously to strain A mice. A single injection of 0.25 cc., containing 2.5 mg. of unextracted dust, was injected in the lateral tail vein; 20 to 30 mice were employed for each specimen.

Extracted Tars

Tricaprylin was used as the vehicle in all cases except for the material from Cambridge which was dispersed in lard filtrate. The extracted tars were injected in amounts which varied from 21 to 71 mg. Table 2 gives the data on the amount of tar used in each instance as well as the weight of dust from which it was derived. A single subcutaneous injection of 0.25 cc. of the tar-containing dispersion was given in the right axilla. For each tar specimen 40 mice were employed, viz, 20 C3H males, 10 A females, and 10 A males. In the case of the Cambridge tar, only C3H male mice were employed; 15 mice received 1 injection of 25 mg. of tar contained in 0.25 cc. of lard filtrate, and 10 other mice received 10 fortnightly injections of 0.1 cc. each of the same preparation.

Controls

A single subcutaneous injection of 0.25 cc. of tricaprylin was given to each of 20 C3H male mice as a control for the effect of the vehicle alone on the induction of subcutaneous sarcomas. A group of 30 untreated strain A mice was kept for a determination of the incidence of spontaneous pulmonary tumors.

Procedure

The strain A mice that had received the dusts intravenously were killed 4 months later. The lungs were examined for the presence and the number of pulmonary tumors (*14*) and compared with the lungs of 20 untreated strain A mice of the same age.

The animals that had received material subcutaneously were examined twice a month or oftener for the presence of progressively growing masses at the site of injection. Post-mortem examination was made of all mice. The incidence of pulmonary and hepatic tumors was determined by gross examination. The nature of the subcutaneous and lymphoid tumors and of representative pulmonary and hepatic tumors was established by microscopic examination.

The experiments were terminated 12 months after the initial procedures, at

Table 2.—*Production of subcutaneous sarcomas in mice by injection of tars extracted from atmospheric dust*

Source of dust	Collection method	Tar injected (per mouse)	Dust equivalent	Mice	
				Effective total	With sarcoma (after 1 year)
		Mg.	*Mg.*	*Number*	*Number*
Chelsea	Filter bag	53	[2]	38	2
Do	Electric precipitron	21	470	39	0
Pittsburgh	do	41	420	32	4
Do	Filter bag	47	[2]	33	5
Holland Tunnel:					
New York intake	Labyrinth	25	900	30	0
New York exhaust	do	48	450	41	1
New Jersey intake	do	55	1,080	33	0
New Jersey exhaust	do	71	520	34	2
Cambridge	Filter bag	25	[2]	11	4
		[1] 100	[2]	6	2

[1] Given in 10 fortnightly injections.
[2] Weight of dust not obtainable with accuracy. (See p. 157.)

which time the mice were 14 to 15 months old.

RESULTS

Sarcomas at Injection Site

Sarcomas at the site of injection began to appear in 5 months. After 6 and 9 months, induced sarcomas were present in 4 and 14 mice, respectively. During the course of a year sarcomas were obtained in 20 mice of an "effective total" (*15*) of 297 (table 2). Sixteen of these twenty sarcomas were induced in an effective total of 171 males of the C3H strain (9 percent). Four sarcomas were induced in an effective total of 126 strain A mice of both sexes (3 percent); all 4 of these sarcomas developed in male mice, although equal numbers of males and females had been employed. Since the material collected in Cambridge represented the dust in the intake air of chemical laboratory buildings in which chemical carcinogens were being synthesized and since it was conceivable that the exhaust air from these laboratories might have contributed small amounts of carcinogens to the outside atmosphere, separate consideration should be given to the results obtained with this particular material.

With regard to the specimens other than that from Cambridge, 10 sarcomas were obtained in an effective total of 154 males of the C3H strain (7 percent) as contrasted with 4 sarcomas in 126 strain A mice of both sexes (3 percent). However, since all of the latter sarcomas occurred in the males which represented half of the strain A mice, the incidence of induced sarcomas in the strain A males was approximately the same as in the C3H males.

Examination of table 2, which summarizes the results on sarcoma production, shows that 6 of the 9 tar specimens yielded 1 or more sarcomas. The tars collected from the street air in Pittsburgh produced sarcomas in 9 mice of an effective total of 65. The rate of production of these sarcomas and the survival data are given in table 3.

Ulceration at the injection site was most pronounced 1 to 2 months after the administration of the tars. At that time there was extensive ulceration in 10 of 140 strain A mice (7 percent) and in 5 of 158 strain C3H mice (3 percent). These lesions healed 1 to 3 months later, at the time tumors began to appear.

The tumors were all pleomorphic spindle-cell sarcomas, indistinguishable from the tumors elicited with polycyclic carcinogens at the site of subcutaneous injection. Many of the tumors contained cysts or particles of the injected material.

No local sarcomas were obtained in the C3H males that had received 20 mg. of unextracted dust subcutaneously. Of these 120 mice, 101 were living at 10 months and 60 at the termination of the experiment after a year. Subcutaneous injection of 0.25 cc. of tricaprylin alone did not elicit tumors at the injection site in any of the 20 C3H males, although 16 of these mice were alive at the end of a year.

Pulmonary Tumors

The data on the incidence of pulmonary tumors in strain A mice 4 months after the intravenous injection of 2.5 mg. of untreated dust are summarized in table 4. Under the conditions of this experiment, the incidence of tumors of the lung was not increased over that obtained in the control animals. Particles of black dust were seen grossly in the lungs of all the injected mice. Only a single tumor was seen in each of the positive mice with the exception of one of the mice that had received dust from Chelsea; this animal had two tumors of the lung.

Table 4 also gives the incidence of pul-

TABLE 3.—*Rate of sarcoma production, and survival data, following subcutaneous injection of tars from air dust*

Source of dust	Collection method	Mice			Total number of mice with sarcoma (+) and negative surviving mice (−), after various periods		
		Number	Strain	Sex	6 months	9 months	12 months
Chelsea	Filter bag	20	C3H	M	0+ 18−	2+ 11−	2+ 1−
		20	A	M+F	0+ 20−	0+ 15−	0+ 11−
Do	Electric precipitron	20	C3H	M	0+ 19−	0+ 14−	0+ 8−
		20	A	M+F	0+ 19−	0+ 16−	0+ 12−
Pittsburgh	do	20	C3H	M	0+ 16−	1+ 11−	1+ 7−
		20	A	M+F	2+ 9−	3+ 4−	3+ 3−
Do	Filter bag	20	C3H	M	0+ 20−	3+ 11−	5+ 4−
		20	A	M+F	0+ 13−	0+ 8−	0+ 5−
Holland Tunnel:							
New York intake	Labyrinth	20	C3H	M	0+ 19−	0+ 18−	0+ 17−
		20	A	M+F	0+ 9−	0+ 8−	0+ 6−
New York exhaust	do	20	C3H	M	0+ 20−	0+ 15−	0+ 9−
		21	A	M+F	0+ 21−	1+ 16−	1+ 16−
New Jersey intake	do	20	C3H	M	0+ 20−	0+ 14−	0+ 7−
		20	A	M+F	0+ 13−	0+ 12−	0+ 11−
New Jersey exhaust	do	20	C3H	M	1+ 17−	2+ 14−	2+ 8−
		20	A	M+F	0+ 16−	0+ 14−	0+ 9−
Cambridge	Filter bag	15	C3H	M	1+ 10−	2+ 9−	4+ 7−
		10	C3H	M	0+ 6−	0+ 5−	2+ 3−

monary tumors in the strain A mice which had received a single subcutaneous injection of air dust tar. These data summarize the results obtained with the mice that died during the twelfth month or that were killed at the end of a year. All of the nine mice that had received tar from Pittsburgh dust bore pulmonary tumors; of these nine mice, seven had two to six tumors of the lung per animal while in the lungs of two mice there were solitary tumors. Whether the material injected was responsible for the increased incidence as compared with the controls cannot be decided from this small number of mice. The incidence of pulmonary tumors in the mice of all the other groups was not significantly higher than in untreated strain A mice in this or in previous experiments (*16*).

These essentially negative findings were duplicated in the results obtained with the C3H male mice that were killed at the termination of the experiments (table

TABLE 4.—*Incidence of pulmonary tumors in strain A mice 4 months after single intravenous injection of 2.5 mg. untreated air dust and 12 months after single subcutaneous injection of about 50 mg. tar from air dust*

Source of dust	Collection method	Dust (intravenous)		Tar from air dust (subcutaneous)		
		Mice killed	Mice with pulmonary tumor	Mice examined	Mice with pulmonary tumor	Average number of tumors per positive mouse
		Number	*Number*	*Number*	*Number*	
Chelsea	Filter bag			11	9	1.3
Do	Precipitron	26	3	16	13	1.4
Pittsburgh	do	27	3	3	3	2.3
Do	Filter bag			6	6	3.5
Holland Tunnel:						
New York intake	Labyrinth	26	0	6	3	1.3
New York exhaust	do	20	5	16	9	1.3
New Jersey intake	do	19	1	11	5	1.8
New Jersey exhaust	do	20	3	9	3	1.3
Controls		20	3	10	5	1.2

5). The incidence of pulmonary tumors in those that had received the unextracted dust subcutaneously was 8 percent; in those that had received air dust tar subcutaneously it was 6 percent. These values are no higher than the incidence of spontaneous pulmonary tumors in untreated mice of a subline of C3H mice (*17*).

HEPATOMAS

The incidence of hepatomas in the C3H mice that died during the twelfth month or that were killed at the end of a year, when they were 14 to 15 months old, is given in table 5. Of the 60 mice that had received 20 mg. of unextracted dust subcutaneously, 8 had hepatomas (13 percent). Of the 81 mice that had received, subcutaneously, air dust tar, 21 had hepatomas (26 percent). Two of the sixteen mice injected with tricaprylin alone had hepatomas (13 percent).

The incidence of hepatomas in the mice injected with tar was, therefore, double that found in untreated controls and in mice that had received unextracted dust. A 26-percent incidence of hepatomas in these C3H mice is not, however, greater than in male C3H mice of the same age in a

TABLE 5.—*Incidence of pulmonary tumors and of hepatomas in C3H male mice 12 months after the subcutaneous injection of untreated air dust or of air dust tar*

Source of dust	Collection method	Unextracted dust (20 mg.)			Tar (21–71 mg.) from air dust in tricaprylin		
		Mice examined	Mice with pulmonary tumor	Mice with hepatoma	Mice examined	Mice with pulmonary tumor	Mice with hepatoma
		Number	*Number*	*Number*	*Number*	*Number*	*Number*
Chelsea	Filter bag				8	1	2
Do	Precipitron	12	1	0	10	1	4
Pittsburgh	do	10	0	2	7	0	1
Do	Filter bag				6	1	2
Holland tunnel:							
New York intake	Labyrinth	11	1	1	11	1	3
New York exhaust	do	7	1	0	17	0	2
New Jersey intake	do	16	2	3	11	0	4
New Jersey exhaust	do	4	0	2	11	1	3
Tricaprylin control					16	0	2

subline maintained at the National Cancer Institute (*17*), or in C3H male mice from Bar Harbor in which the age ranged between 12 and 19 months (*18, 19*). On the basis of the data available at present, the increased incidence of hepatomas in the test mice is not considered significant.

Other Neoplasms

Three female strain A mice developed mammary adenocarcinomas. These three mice were all in different groups, which had received tar from dusts obtained from the precipitron in Chelsea, and from the New York intake and the New Jersey exhaust air of the Holland Tunnel. The tumors appeared 6, 7, and 9 months, respectively, after the start of the experiment.

Three C3H male mice, two of which had received tar from the intake and exhaust air dust, respectively, of the New Jersey side of the Holland Tunnel, and one of which had received untreated dust from the New York exhaust air, developed lymphoid tumors 5, 6, and 8 months following injection. These three mammary gland tumors and three cases of lymphoid tumor presumably did not result from the experimental treatment and are, provisionally, considered to be spontaneous in origin.

DISCUSSION

Previous workers with animals have utilized several approaches to the problem of the possible carcinogenic activity of materials in the air, or of materials which are common sources of air pollution. Passey (*4, 5*) obtained carcinoma of the skin in mice painted with tar extracted from household chimney soot. However, Leitch (*20*) obtained negative results on rubbing chimney soot into the scrotum of rabbits thrice weekly for 2 years and into the scrotum of rats for 1 year. Campbell (*6*) collected sweepings of dust from tarred roads and exposed mice to the dust resuspended in a respiratory chamber. Cutaneous tumors developed in the mice; they also showed a higher incidence of pulmonary tumors than did the control mice. He (*7*) found that inhalation of exhaust material from gasoline motors did not induce pulmonary tumors, nor did the exposed mice develop cutaneous umors. Campbell (*8*) reported that exposure to tarred road dust, from which most of the tar had been extracted, did not produce cutaneous tumors but did increase the incidence of pulmonary tumors. In subsequent experiments (*9*), he exposed mice to suspensions of "soot from a domestic chimney ventilating an ordinary coal fire," and to soot from the exhaust of a motor vehicle burning heavy oil; in both cases negative results were obtained as regards production of tumors of the skin, and no significant increase in the incidence of tumors of the lungs resulted. When the extracted tars were painted on the skin of mice, no cutaneous tumors were obtained with the tar from exhaust soot, while the tar from chimney soot was found to be carcinogenic for the skin. More recently Campbell (*10*) concluded that exposure of mice to "dusting" with precipitated silica or brown iron oxide resulted in a significant increase in the incidence of primary tumors of the lung in mice living 10 months or longer. However, since the mice were stated to be of "mixed strain," it would be of interest to have similar data with mice of an inbred strain in which more extensive information is available on the incidence of pulmonary tumors in untreated control animals.

Seelig and Benignus (*21*) worked with soot from a chimney burning bituminous coal. The soot was used as bedding for the mice (Buffalo strain). Primary tumors of the lung were obtained in 8 of the 100 test mice as compared with 1 of the 50 control mice (2 percent). In their next

experiment they (*22*) employed C57 Black mice and a gas-works tar of known carcinogenic potency which was deposited on tar-free lampblack. Inhalation of this material did not produce pulmonary tumors in any of 100 test mice, whereas 2 of the 50 control mice had primary tumors of the lung.

Shimkin and Leiter (*23*) extracted the tar from chimney soot and injected it subcutaneously into mice; sarcomas were obtained in about half of the animals. These results were in agreement with those of Passey and of Campbell with regard to the potency of tar from chimney soot. Intravenous injection, in strain A mice, of the extracted soot did not yield a higher incidence of primary pulmonary tumors than in the control mice, whereas similar injection of the unextracted soot gave evidence of an increased incidence of primary tumors of the lung.

The collection of dust specimens for the purpose of these experiments presents a number of problems. In the first place, the size of many of the particles in street air dust is so small that highly efficient apparatus is required if loss of a large proportion of the finer particles is to be avoided. The diameter of about half of such particles is less than 0.5μ and of about 97 percent is less than 1μ (*24*). Most industrial dusts, which are the types that have been usually studied in dust investigations, have particle sizes considerably larger.

Secondly, relatively large amounts of dust are required for animal experiments in which even only a single dose of 50 mg. of tar is given. The types of collectors ordinarily employed in dust analyses are inadequate for these biochemical studies inasmuch as the amount of dust in street air varies between about 2 and 7 mg. per 300 cubic feet (*25*) and the capacity of laboratory dust collectors is usually less than 3 cubic feet per minute (*24*). The use of the filtering equipment in commercial air-conditioning installations is also not generally suitable, since these filters usually involve a wet method in which the filter material is impregnated with a liquid, usually oil, to increase retentivity of the particles. In an exploratory experiment, one such filter was extracted, but it was found that the large amount of oil used for impregnation resulted in an undesirably high dilution of the tar. Even with electrical precipitation, the large-scale installations usually employ oil, which is sprayed on the precipitating plates to promote better adhesion of the discharged dust particles.

In this investigation the purpose was to ascertain qualitatively whether evidence of carcinogenic activity could be obtained. Accordingly, no quantitative estimations of efficiency of collection were made. However, certain general questions of efficiency were considered.

Of the collectors used in the present experiments, the precipitron was probably the most efficient, since the limits to its efficiency are the rate of air flow, and the size of and potential gradient in the collector cell, all of which can be varied to give any desired efficiency. However, the use of oil on the condenser plates was deliberately avoided; consequently there were probably losses due to blowoff of some of the adherent dust. One of the possible disadvantages of electrical precipitation, especially when the plates are not oiled, is the danger of excessive ozone formation. Since oxidized carcinogenic hydrocarbons and oxygen-containing derivatives of such hydrocarbons have been found to possess less potency than the parent compounds or none at all (*26*), an excessive concentration of a powerful oxidant might be objectionable. It was for this reason that a mechanical filter was set up in parallel

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with the precipitron, in spite of the fact that mechanical filters such as cotton flannel have a low efficiency since they retain only the larger dust particles; it is only as the pores become plugged with retained larger particles that their efficiency progressively increases.

Labyrinths such as those used in the Holland Tunnel system also have low efficiencies for the smaller particles. Here, too, it is chiefly the larger particles that are collected.

In the previous work of Siegel and Shear with sedimented air dusts, about 50 mg. of tar was injected in 7 divided doses over a period of 11 months. In the present work, with but one exception, approximately the same amount of air dust tar was given in a single injection; the amounts varied from 21 to 71, with an average of 48 mg. This resulted in the production of sarcomas in 18 of an effective total of 291 mice during a period of 12 months, which represents an over-all incidence of 6 percent. However, negative results were obtained with 3 specimens, and only 1 or 2 tumors were obtained with 3 other specimens. It was only with the Cambridge tar and the 2 Pittsburgh tars that a higher yield of sarcomas was obtained, but even in these 3 instances the number of mice with induced sarcomas was only 13 in an effective total of 76 mice, or an incidence of only 17 percent. While definitely positive results were obtained as regards carcinogenic potency, the tumor-producing potency of these materials was nevertheless low in these experiments.

Attention is called to the fact that the dose employed was a single injection of approximately 50 mg. of extracted tar. Fractionation of the tar might concentrate in some fraction the presumably minute amounts of carcinogen and thus give a more potent material. For these reasons, we do not consider that the specimens which gave no tumors in these experiments have been demonstrated to be noncarcinogenic. If procedures were developed which would provide fractions of high potency from certain specimens while analogous fractions from other specimens were completely inactive, the contrast would be more significant.

From table 1 it is seen that at the New York end of the Holland Tunnel the exhaust air dust contained more tar than the dust in the intake air; the same situation held true for the materials collected at the New Jersey end. Tumors were obtained with the exhaust dust tar from both ends of the tunnel, whereas both specimens of intake dust tar gave negative results. The significance of these contrasting results may, however, be apparent rather than real, since the potency of the active materials was so low. Moreover, no attempt was made in these qualitative experiments to administer precisely equal amounts of tar.

As stated previously, these experiments were restricted to the mouse, and our interpretations are, for the present at least, also restricted to this experimental animal. Even in the case of the mouse, this work provides no information on the production of pulmonary tumors by inhalation of these dusts. It does show, however, that tars obtained from these dusts are carcinogenic for the subcutaneous tissue of the mouse.

SUMMARY

Nine specimens of atmospheric dust were obtained from various locations. The collection methods employed were: cloth filter bags in Chelsea and Cambridge, Mass., and Pittsburgh; electric precipitation in Chelsea and in Pittsburgh; and labyrinth precipitation in the case of both intake and exhaust air at the New York and New Jersey ends of the Holland Tunnel.

Subcutaneous injection, into male C3H mice, of 20 mg. of unextracted dust (six specimens) in saline, or of 0.25 cc. of tricapyrylin alone, did not produce sarcomas at the site of injection in 12 months.

A single subcutaneous injection of about 50 mg. of tar, extracted from these dusts by benzene and by ethyl ether and dispersed in tricaprylin, produced sarcomas at the site of injection in 18 of an effective total of 291 mice (strain C3H males and strain A mice of both sexes) in 12 months.

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Production of Tumors in Mice With Tars From City Air Dusts[1]

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Siegel and Shear[2] found that subcutaneous injection of about 50 mg., in 7 divided doses, of tar extracted from sedimented air dust from New York City did not produce sarcomas in mice. Subsequently it was found (*2*) that a single injection of about the same amount of tar, extracted from atmospheric dust collected by various techniques in 5 cities, produced sarcomas in 18 of an effective total of 291 mice.

The collection methods employed were not considered ideal. Filtration of the air through cotton cloth and mechanical precipitation in a labyrinth were considered relatively inefficient, particularly as regards the smaller particles. Electrical precipitation on unoiled plates was a more efficient collection method, but the formation of ozone and possibly of nitrogen oxides in the precipitron was considered undesirable since it was conceivable that some of the collected material might undergo oxidation.

A collector was therefore designed which was free from both of these objections. Air from the street was drawn through the collector and retained on filter paper selected for this particular purpose. Units of these large-capacity collectors were set up in five industrial cities in locations selected from the point of view of representing general conditions rather than those peculiar to any special industrial location. In addition, dust specimens were obtained from three other cities by methods described in the preceding communication.

The black dusts were extracted with reagent-grade benzene, and the tars obtained were dispersed in tricaprylin. A single subcutaneous injection of about 50 mg. tar, representing about 0.5 gm. of dust, was given to C3H mice. As before, the first tumor appeared in 5 months. At the end of a year, tumors had been produced at the injection site in 22 mice. At 16 months induced tumors had been obtained in 30 of an effective total of 372 mice, an incidence of 8 percent.

FIGURE 1.—External view of the large-capacity air-dust collector, showing the inlet duct with the open end protected by a screen of wire gauze, and the louvers for the discharged air.

[1] *Presented* (1) *at the Boston meeting of the American Association for Cancer Research, March 31, 1942.*

[2] *See footnote 1, p. 1, of preceding paper* (2).

FIGURE 2.—Top view of the air-dust collector prior to insertion of filter paper, showing the upper and lower sets of rods.

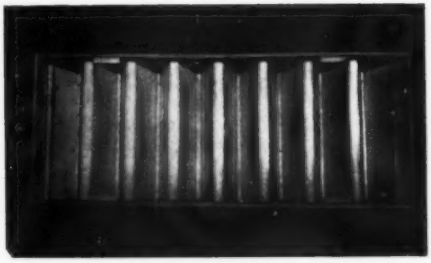

FIGURE 3.—Top view of the air-dust collector showing the paper convoluted between the upper and lower sets of rods, and the upper side frames holding the side edges of the paper in position.

APPARATUS AND METHODS

The cloth-filter-bag apparatus and the electrical precipitron, described previously (*2*), were employed in Pittsburgh. Another precipitron was installed in East St. Louis, Ill. The dust specimens from the labyrinths of the Holland Tunnel were obtained as before from the intake and exhaust air at both the New York and New Jersey sides of the tunnel.

A new type dust collector was designed and installed in Chicago, Cincinnati, Youngstown, and East St. Louis, and in South Charleston, W. Va. The apparatus consisted of a large wooden box (fig. 1) containing an electric motor-driven blower of high capacity which drew air from the street over and through a large sheet of special filter paper[3] held in place by supporting rods (figs. 2 and 3).

The paper had a low resistance to air flow and a high efficiency for the retention of very small particles. This was determined in experiments with dust suspensions composed of particles with estimated diameters less than those of air dust particles. The resistance to air flow was well below that specified for the rated capacity of the blower, which was 275 cubic feet of air per minute at 4 inches of static pressure. The blower was operated by a one-half-horsepower motor.

Air from the street was drawn into the box through a duct. It then passed through a single sheet of filter paper 2 feet wide and 17 feet long. In order to accommodate this length in a box 5 feet long, the paper was convoluted between two sets of rods (figs. 2 and 3). Figure 4 is a diagrammatic sketch of the apparatus. The side edges of the paper were supported between pairs of wooden boards cut in a sawtooth pattern. The lower boards were fixed to the sides of the box while the upper ones were removable to permit insertion and removal of the paper. The lower edges of the removable boards were cut so as to fit into the "teeth" of the upper edges of the fixed boards, with just enough intervening space to permit lining of the edges of the upper and lower boards with felt. On clamping the edges of the paper between the felted edges of the wooden supporting boards, an air-tight seal was obtained. The end edges of the paper were clamped between felt-lined boards along the ends of the box to complete the air-tight seal. In this manner 34 square feet of filtering surface were ob-

[3] *The apparatus was designed with the cooperation of Col. M. E. Barker and L. J. Rose, of the Chemical Warfare Service, United States Army. The specifications for the paper were also obtained from the same source. Tests on the properties of the paper were performed at the Edgewood Arsenal. Since this paper is a restricted material, details of the tests cannot be given here.*

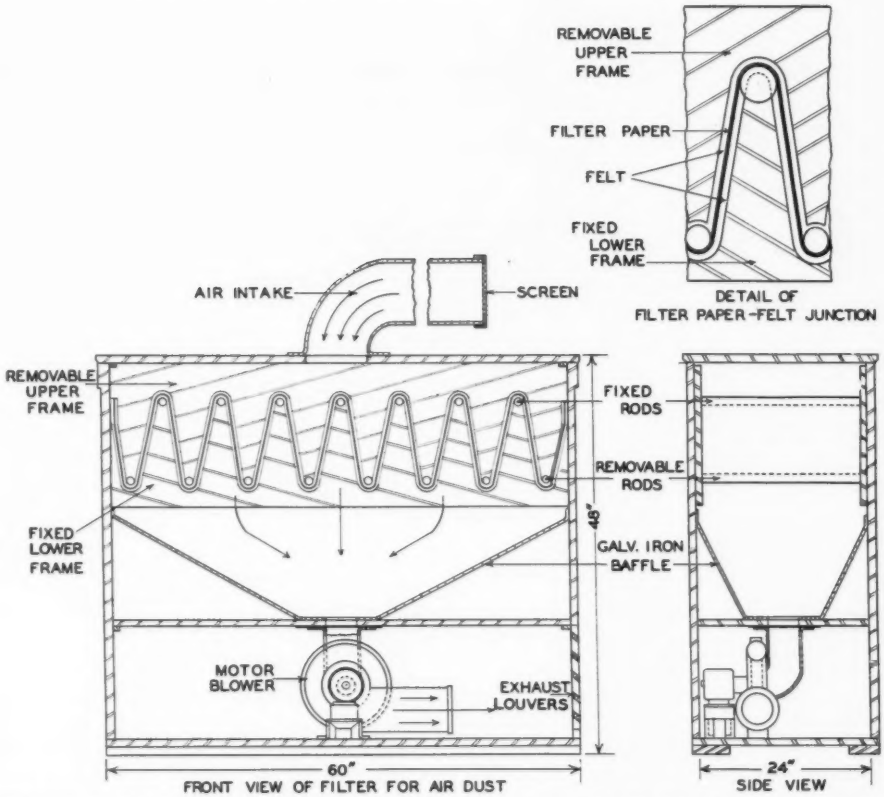

FIGURE 4.—Diagrammatic sketch of the high-capacity filter apparatus for the collection of air dust.

tained in a unit 5 feet long and 2 feet wide.

The air passing through the paper was directed to the inlet of the blower, located in the lower part of the box, by a galvanized iron baffle. The filtered air was discharged through louvers in the lower part of the box.

To minimize the entrance of rain water into the inlet tube, the free end was cut at an angle so that the upper edge protruded. The opening was covered by a wire-gauze screen to keep out insects, paper, etc.

The cities selected for installation of the new dust collectors were chosen from among those having a high concentration of industrial plants and having smoke-polluted air. In three instances these cities were located in valleys or were surrounded by bluffs, which tended to retard ventilation of the air above them. In all cases the cities were in the immediate vicinity of a river or lake, so that the moisture contributed to the formation of smog.

The locations [4] of the five newly de-

[4] *Dr. I. Arthur Mirsky, director of the May Institute for Medical Research of the Jewish Hospital of Cincinnati, Dr. E. V. Cowdry, director of research of the Barnard Free Skin and Cancer Hospital, St. Louis, and I. A. Deutch, Department of Smoke Inspection and Abatement, Chicago, cooperated in the selection of the sites in or near their respective cities and in the maintenance of continuous operation of the collectors.*

signed dust collectors, and of the older ones in Pittsburgh, were as follows:

Chicago.—The roof of the 12-story City Hall, which is in the heart of the Loop, the business district of the city.

Cincinnati.—The fifth floor of the Ohio State National Guard Armory, on Freeman Avenue, which overlooks railroad yards and the Union Station.

East St. Louis.—The basement of the Post Office Building on Missouri Avenue, which is near railroads and large manufacturing plants; the inlet of the duct was about 3 feet above the street level.

South Charleston.—The municipal garage which is a short distance from large factories and near busy railroad tracks; the inlet of the duct was about 6 feet above the ground.

Youngstown.—The third floor of the Post Office Building, at Front and Market Streets, which is in the heart of the business and manufacturing district and close to several busy railroad lines.

Pittsburgh.—The cloth-filter-bag collector and a precipitron were placed on the second floor of the Post Office Building, at 7th and Grant Avenues, which is about 1 block from a large railroad station in the heart of the downtown business district.

After continuous operation of the apparatus for 2 or 3 months, the paper was removed and was replaced with a fresh sheet. The dust, which had collected in a thin layer over the entire surface of the paper, could be almost completely removed by gentle scraping, with no appreciable contamination by paper fibers.

The dust specimens were then treated in a large Soxhlet extractor with reagent-grade benzene in the manner described previously (*2*). The extracted tars were dispersed in tricaprylin and injected subcutaneously into mice. A single injection of 0.25 cc., containing between 41 and 67 mg. of tar, was given (May 22, 1941) in the right axilla. Between 20 and 32 male mice of the C3H strain were employed for each specimen of tar. A total of 400 mice received 14 tar specimens.

All of the mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. They were about 3 months old at the start of the experiment. Unlimited amounts of Purina dog chow and of tap water were available at all times.

RESULTS

The weights of the dust specimens taken for extraction and the yields of tar are given in table 1. This table also gives the volume of tricaprylin employed for dispersion of each tar specimen and the number of mice employed for injection in each group. Of the four specimens from Pittsburgh, two were freshly extracted tars from newly collected dust samples; the other two were aliquots of the same tar specimens that had been injected into mice a year previously. In agreement with our earlier findings, the dusts from exhaust air of the Holland Tunnel had a higher content of tar than had the dusts from intake air.

Table 2 summarizes the results on the production of tumors at the site of injection. Tumors were obtained in 22 of the mice by the end of a year. Of the original 400 mice injected at the start of the experiment, 372 were alive at the time of appearance of the first sarcoma at the end of the fifth month. Thus, of the effective total of 372 mice, 6 percent had developed tumors in a year. Tumors later were produced in 8 more mice, giving an incidence of 8 percent in 16 months. (See table 3.)

In general, the results were similar to those obtained previously. The over-all incidence of induced tumors was low. With the exception of four specimens, the positive tars yielded only one or two tumors per group, while one of the specimens gave negative results. As before, the most potent material was that obtained from Pittsburgh.

The Pittsburgh specimens which gave the most tumors (four and five, respectively) were freshly extracted tars from

TABLE 1.—*Data on air dust specimens and tars extracted from them with benzene*

Source of dust	Collection method	Weight of dust	Weight of tar	Volume of tricaprylin	Mice injected
		Grams	*Grams*	*Cubic centimeters*	*Number*
Chicago	Filter paper	30	2.2	10.1	30
South Charleston	do	35	1.8	9.0	30
Cincinnati	do	25	2.5	9.8	30
Youngstown	do	30	3.4	13.4	30
East St. Louis	do	22	2.4	9.0	30
Do	Precipitron	14	2.0	10.3	31
Pittsburgh	do	35	2.5	12.2	30
Do	Filter bag	(1)	2.3	11.1	30
Do	do.[2]	(1)	2.6	12.1	20
Do	Precipitron [2]	29	2.7	13.0	20
Holland Tunnel:					
New York intake	Labyrinth	55	1.8	7.5	27
New York exhaust	do	18	1.9	10.1	30
New Jersey intake	do	45	2.2	9.3	32
New Jersey exhaust	do	20	2.3	13.9	30

[1] Weight of dust not obtainable with accuracy. (See p. 157 of preceding paper (*2*).)
[2] These were aliquots of the same specimens of tar tested previously (*2*).

dust samples that had been collected recently. The other two specimens (which gave only one tumor in each instance) were aliquots of the same tar specimens that had been injected into mice in the previous year and had given the largest number of sarcomas (four and five, respectively). This raises the question as to whether the carcinogen present in such material undergoes chemical change on long standing, with concomitant loss of potency. On the other hand, it is conceivable that the different values for tumor incidence obtained in the duplicate experiments with aliquots of the same specimens may have been due to chance variation.

One of the specimens from the Holland Tunnel gave negative results; the others gave one or more tumors each. In the previous experiment, two specimens gave negative results while the other two yielded one and two sarcomas, respectively.

The rate of production of tumors at the injection site and survival data are given in table 3. The first tumor appeared after 5 months. By the end of 9 months, tumors had developed at the site of injection in 13 animals. Tumors were obtained in

TABLE 2.—*Production of tumors at injection site in C3H male mice by subcutaneous injection of tars extracted from air dust*

Source of dust	Collection method	Tar injected (per mouse)	Dust equivalent	Mice	
				Effective total	With tumor (after 1 year)
		Mg.	*Mg.*	*Number*	*Number*
Chicago	Filter paper	55	745	27	1
South Charleston	do	50	970	28	2
Cincinnati	do	64	635	27	0
Youngstown	do	63	565	28	2
East St. Louis	do	67	625	23	2
Do	Precipitron	49	330	29	3
Pittsburgh	do	51	720	30	3
Do	Filter bag	52	(1)	27	5
Do	do.[2]	54	(1)	19	1
Do	Precipitron [2]	52	560	19	1
Holland Tunnel:					
New York intake	Labyrinth	60	1,830	27	0
New York exhaust	do	47	445	29	0
New Jersey intake	do	59	1,210	31	1
New Jersey exhaust	do	41	360	28	1

[1] Weight of dust not obtainable with accuracy. (See p. 157 of preceding paper (*2*).)
[2] These were aliquots of the same specimens of tar tested previously (*2*).

TABLE 3.—*Rate of tumor production in C3H mice, and survival data, following subcutaneous injection of tars from air dust*

Source of dust	Collection method	Total number of mice with tumor (+) and negative surviving mice (−), after various periods			
		6 months	9 months	12 months	16 months
Chicago	Filter paper	0+ 26−	0+ 26−	1+ 21−	2+ 18−
South Charleston	do	0+ 27−	0+ 27−	2+ 22−	2+ 14−
Cincinnati	do	0+ 27−	0+ 24−	0+ 15−	2+ 9−
Youngstown	do	0+ 27−	2+ 14−	2+ 11−	3+ 8−
East St. Louis	do	1+ 22−	2+ 18−	2+ 15−	2+ 11−
Do	Precipitron	0+ 26−	2+ 18−	3+ 7−	4+ 3−
Pittsburgh	do	0+ 29−	2+ 23−	3+ 15−	4+ 6−
Do	Filter bag	0+ 25−	4+ 19−	5+ 8−	5+ 4−
Do	do.[1]	0+ 18−	0+ 10−	1+ 10−	1+ 4−
Do	Precipitron [1]	0+ 19−	0+ 19−	1+ 17−	1+ 12−
Holland Tunnel:					
New York intake	Labyrinth	0+ 27−	0+ 27−	0+ 27−	0+ 25−
New York exhaust	do	0+ 29−	0+ 28−	0+ 28−	1+ 23−
New Jersey intake	do	0+ 31−	0+ 31−	1+ 28−	2+ 25−
New Jersey exhaust	do	0+ 28−	1+ 25−	1+ 18−	1+ 16−

[1] These were aliquots of the same specimens of tar tested previously (*2*).

a total of 22 mice by the end of a year. At 16 months, the total number of mice with induced tumors was 30.

Of the 30 tumors induced at the site of injection of these tars in male mice, 26 were sarcomas. Four were squamous-cell epithelial tumors; of these, three were carcinomas and one was a papilloma. These results parallel those obtained on subcutaneous injection of carcinogenic compounds, in which carcinomas may be obtained in some mice whereas most of the tumors are sarcomas.

Survival was quite satisfactory. In 1 experiment, all 27 of the mice were alive at the end of a year. In 3 other groups only 2, 3, and 4 mice died during the course of the experiment. Of the original 400 mice, 242 were alive and negative at the end of a year. There were 178 negative survivors at 16 months, when the experiment was ended.

All of the dust specimens were black, with the exception of the dust from South Charleston, which was dark gray. The extracted tars were all black.

DISCUSSION

The previous finding (*2*) that tars extracted from city air dust produced sarcomas in mice at the site of injection has been confirmed. The technique, a single subcutaneous injection of about 50 mg. of

tar dispersed in tricaprylin, was again employed and yielded similar results.

Of the 14 specimens tested, 13 produced tumors in 1 or more mice. Tumors were obtained in 30 of an effective total of 372 mice, an incidence of about 8 percent of all mice. These figures are in good agreement with those obtained previously.

The qualitative data presented here do not afford a basis for classification of cities according to the cancer-producing potency of the contaminants in their respective atmospheres. The results show qualitatively that it is possible to extract from the dust in the air in some cities a material which is carcinogenic for the mouse. Although the work of Passey, Cambell, and others indicated that dust in the atmosphere might well contain carcinogenic material, inasmuch as they found that substances [5] likely to contaminate the air were carcinogenic, the present and preceding paper provide direct evidence on the presence of carcinogenic tar in the air itself.

Roffo (*5*) reported that filtration of the "town air" of Buenos Aires through air purifiers gave a mineral oil with absorption and fluorescence spectra characteristic for certain unsaturated polycyclic hydrocarbons. No animal experiments were reported, and the identity of the spectra with those of any carcinogenic hydrocarbon was not established. McDonald and Woodhouse (*6*) have recently reported experiments in which mice were treated with atmospheric dusts and tars derived from them. One sample (plenum dust) was obtained from the air-purifying screens of a hospital in close proximity to factories; the other was sedimented dust from city thoroughfares. Mice exposed to inhalation of these dusts for a year or more had adenomas of the lung in 23 and 5 percent, respectively, of the survivors as compared with an incidence of 16 percent in the untreated mice. Inasmuch as "market mice" were used, it is uncertain whether a difference between 23 and 16 percent is to be considered significant. Moreover, the authors stated (*6, p. 11*):

> The number of surviving animals at the end of the experimental period is too small to permit of statistical analysis of the lung tumour incidence. This preliminary investigation appears to indicate that the adenoma incidence in the plenum dust group does not show the striking increase over the control incidence obtained by Campbell (1934) in a similar experiment with dust from tarred roads.

The tar was extracted by McDonald and Woodhouse from the plenum dust and distilled under reduced pressure. One fraction was painted on the skin of mice for a year. No statement was made with regard to the production of any skin tumors. The incidence of pulmonary tumors in these mice was 25 percent as contrasted with 16 percent in the controls. A fraction obtained in fractional distillation, in which temperatures as high as 300° were employed, yielded an absorption spectrum which, according to these authors, "gave evidence of the presence of benzpyrene." Inspection of the curve, however, shows that the maxima were shifted towards the ultraviolet end of the spectrum as compared with the curve for benzpyrene. While the background absorption might have been responsible for this shift, it is also conceivable that the specific absorption might be due to some compound closely related to 3, 4-benzpyrene rather than to benzpyrene itself. In any case, it was tar treated at elevated temperatures, and not atmospheric dust, in which benzpyrene was stated to have been demonstrated.

[5] *For references to earlier work, see the preceding paper* (2). *While it was stated* (3) *that Passey "demonstrated the presence of compounds carcinogenic to mice" in materials "discharged into the air", he had employed chimney soot and not material from the air. He found that tar extracted from the soot was carcinogenic, but the presence of individual compounds was not demonstrated.*

Our animal experiments have established that tars extracted at low temperature from city air dusts are carcinogenic for the mouse. While it is conceivable that the carcinogen present may be 3, 4-benzpyrene, and while the interesting spectra in the paper of McDonald and Woodhouse point in this direction, further data are required before this matter can be considered established.

In this connection, a ton of air dust was obtained from the labyrinths of the Holland Tunnel. Low temperature extraction with benzene yielded about 200 pounds of tar.[6] It is planned to attempt to fractionate this material without using the high temperatures required for fractional distillation.

While these air-dust studies were designed to obtain information on factors which may contribute to the genesis of primary pulmonary tumors in man, it is again pointed out that the experiments performed were restricted to the mouse and that the conclusions drawn from the data are also restricted to this animal.

SUMMARY

A new dust collector was designed, with a capacity of 275 cubic feet of air per minute, in which a single sheet of paper 17 feet long and 2 feet wide was the filter. The paper had a low resistance to air flow and a high efficiency for retention of very small particles.

A unit of this collector was installed in each of the following cities: Chicago, East St. Louis, Cincinnati, Youngstown, and South Charleston, W. Va. In addition, a cloth-filter-bag apparatus and an electric precipitron were employed in Pittsburgh, and another electric precipitron in East St. Louis. Samples of dust from the intake and exhaust air of both the New York and New Jersey ends of the Holland Tunnel were obtained from the labyrinths of the ventilating system of the tunnel.

Continuous operation of the collectors provided specimens of black dust which yielded black tar on extraction with benzene. The tars were dispersed in tricaprylin and injected subcutaneously in 400 male mice of the C3H strain. A single injection of about 50 mg. of tar, dispersed in 0.25 cc. of tricaprylin, was given.

The first sarcoma appeared at the injection site after 5 months at which time there were 372 survivors. By the end of 16 months tumors had been obtained in 30 mice, which represented an incidence of 8 percent of the effective total

These results confirm and extend those obtained in the preceding study.

[6] *Hartwell, J. L., and Shear, M. J.: Unpublished work (1941).*

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Adenocarcinoma of the Pyloric Stomach and Other Gastric Neoplasms in Mice Induced With Carcinogenic Hydrocarbons[1]

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INTRODUCTION

For a number of years experiments have been in progress in this Institute dealing with the induction of tumors of the gastrointestinal tract. The carcinogenic hydrocarbons[2] used, 1, 2, 5, 6-dibenzanthracene, 20-methylcholanthrene, or 3,4-benzpyrene, were either injected into the wall of the stomach or intestines or administered orally (*1–5*). So far squamous-cell carcinoma of the forestomach and adenocarcinoma of the small intestine have been obtained and reported. The present paper deals with the induction of adenocarcinoma of the glandular stomach and other gastric tumors in mice by the injection of carcinogenic hydrocarbons into the wall of the pyloric stomach. In the literature dealing with the induction of adenocarcinoma of the stomach in animals (*1, 6*), the opinion has been expressed that there is no reliable method of inducing adenocarcinoma of the stomach in animals. In fact, there is no well-established case of an adenocarcinoma of the stomach produced experimentally prior to the preliminary report of the present work (*5*).

MATERIALS AND METHODS

The data on the number, strains, and sex of the mice, the dose of the carcinogenic hydrocarbon used, as well as the vehicle, are given in table 1.

In one experiment threads were coated with the carcinogenic hydrocarbon by the technique described by Andervont (*7*). A piece of white cotton thread size 60 (Clark's) was dipped in the melted hydrocarbon so that the tip of the thread was coated with approximately 0.5 mg. of the material. The thread was then stitched into the anterior wall of the pyloric stomach leaving the coated tip buried in the tissue, and the remaining thread was cut away. The length of the buried piece of thread was usually such that it reached from a point near the limiting ridge to a level approximating the pyloric ring.

In the other experiments the carcinogenic hydrocarbon was injected into the wall of the pyloric stomach by means of a tuberculin syringe and a 27-gage needle. The following preparations were used: (1) A mineral-oil solution containing 6.8 mg. of methylcholanthrene per cubic centimeter; (2) a lard solution containing 4 mg. of dibenzanthracene per cubic centimeter; and (3) a dispersion of methylcholanthrene in horse serum containing 20 mg. of the hydrocarbon per cubic centimeter. The method for preparing the methylcholanthrene dispersion has been published (*5*). The melting points of the polycyclic carcinogenic hydrocarbons employed were as follows: benzpyrene, 178.2°–178.9° C.;

[1] *Presented at the 35th annual meeting of the American Association for Cancer Research, Boston, March 31 and April 1, 1942.*

[2] *Hereinafter referred to as dibenzanthracene, methylcholanthrene, and benzpyrene, respectively.*

TABLE 1.—*Induction of gastric adenocarcinoma and other gastric tumors in mice by the introduction of carcinogenic hydrocarbons into the wall of the pyloric stomach*

Experiment No.	Number of mice	Strain	Sex	Hydrocarbon	Dose	Vehicle	Type of gastric tumor induced							
							Adenoma	Adenocarcinoma	Adenoacanthoma	Adenocarcinoma and sarcoma mixed	Adenoacanthoma and sarcoma mixed	Sarcoma	Squamous papilloma	Squamous carcinoma
					Mg.									
1	6	C3H	M	Dibenzanthracene.	0.5	Thread						1		
2	7	C3H	M	Methylcholanthrene.	.5	do								
3	6	C3H	M	Benzpyrene	.5	do								
4	14	A	M	Methylcholanthrene.	.34	Oil	2							
5	11	A	F	do	.34	do	1						3	
6	16	A	M	do	.2	Serum	2					1	2	
7	11	A	F	do	.41	Oil				1			2	
8	17	C3H	M	do	.41	do	2		1			3	4	3
9	16	C57 Black	M	do	.34	do	3			1		3	8	
10	14	C	M	do	.34	do	2					2	2	2
11	12	C3H	M	Dibenzanthracene.	.2	Lard	4			4		2	1	1
12	47	C3H	M	Methylcholanthrene.	.8	Serum	7	6	6	12	2	3	9	2
13	35	C3H	M	do	.2	Oil	5					9	4	3
14	28	A	M	do	.14	do	2					3	3	1
15	4	I	M	do	.14	do		2						
					.6	Serum								
16	19	I	M	do	.14	Oil	4						1	
17	9	I	F	do	.14	do	3							
18	5	C57 Black	F	do	.14	do	4					1	1	2
19	7	do	M	do	.14	do	1		1			1	2	
20	11	Dilute brown	M	do	.14	do	1						1	
21	2	do	F	do	.14	do	1					1	1	

dibenzanthracene, 267.3°–267.8°; methylcholanthrene, 178.6°–179.6°.

The mice were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, and were 3 to 5 months old when the experiments were begun. Intraperitoneal urethane (ethyl carbamate) was the anesthetic employed. The stomach was delivered through a midline abdominal incision. The needle attached to the syringe was inserted just proximal to the pyloric ring, and the injected material was forced into the anterior wall of the glandular stomach along the lesser or greater curvature. In experiment 15 (table 1), injections were made into both the anterior and posterior walls. The serosal surface of the glandular stomach of the mouse has a somewhat irregular knobby surface so that difficulty was experienced in making the injections, particularly in the earlier experiments. Despite the utmost precaution some of the injected fluid sometimes escaped into the wall of the cardiac chamber. An attempt was always made to inject the carcinogen as near the mucous membrane as possible, but in some animals injected material was observed microscopically beneath the peritoneum and in the muscularis.

The living mice were examined at regular intervals; and if a gastric mass was palpated, the animal was killed and autopsied. In virtually all cases the lumen of the stomach was injected with fixative at autopsy and the specimen hardened before it was sectioned. Acid Zenker's, formol-Zenker's, or dilute formaldehyde solution (formalin U. S. P. 1:10) were used. The tissues were blocked in paraffin and stained with haematoxylin and eosin, phosphotungstic acid-haematoxylin, Laidlaw's silver

stain for reticulum, Mallory's aniline blue connective tissue stain, and van Gieson's connective tissue stain.

RESULTS AND DISCUSSION

Table 1 gives the data on the occurrence of gastric tumors in mice following the introduction of carcinogenic hydrocarbons into the wall of the glandular chamber of the stomach. Two or more gastric tumors coexisted in some of the mice. In all experiments except those with the coated thread, one or more neoplasms composed entirely or in part of glandular epithelial elements arose in at least some of the mice. There were so many variables in the different experiments that it was not possible to make any strict comparisons between the various groups of mice treated. However, with the point in mind that these experiments were designed to establish some method by which adenocarcinoma of the stomach could be induced with some degree of consistency, it appears that experiment 12 fulfills this condition. In this experiment 47 male C3H mice received an injection of 0.8 mg. of methylcholanthrene dispersed in horse serum into the wall of the glandular chamber of the stomach. A total of 47 tumors were induced, 33 of which had glandular elements composing all or a part of the tumor, and 6 were adenocarcinomas. The only other experiment in which adenocarcinoma was obtained was experiment 15 in which 2 of 4 strain I mice showed this lesion.

Table 1 shows that a large number of neoplastic lesions in the mice of different groups were classified as adenoma. No glandular tumor was classified as malignant unless there was infiltration of tumor cells through both muscular coats of the stomach and the neoplastic nodule was present on the peritoneum. Because of this rigid criterion, it is probable that some tumors classified as adenoma were potential carcinomas or perhaps examples of so-called carcinoma *in situ*. This rigid criterion is justified because the literature contains many claims of induction of adenocarcinoma of the stomach in which the pathologic descriptions and illustrations do not support the diagnosis (*6*).

A number of squamous papillomas and squamous-cell carcinomas of the forestomach also occurred in the mice of these experiments (table 1). As mentioned above, despite precautions, small amounts of the injected material sometimes escaped into the wall of the forestomach. It is also considered probable that following injection into the wall of the glandular stomach some of the material may have been worked into the wall of the forestomach as a result of peristaltic action. The occurrence of forestomach neoplasms can thus be explained on either basis. Grossly and microscopically these tumors were similar to those induced in the forestomach of mice by direct injection into the wall of the forestomach or by oral administration of the carcinogenic hydrocarbons (*1*, *4*).

Adenocarcinoma

Table 2 contains the data on induced adenocarcinoma of the pyloric stomach. All the animals had been injected with the horse serum dispersion of methylcholanthrene. In addition, two mice also received an injection of 0.14 mg. of methylcholanthrene in mineral oil. The tumors in these two mice were on the mucosal surface of the stomach at the site of the injected dispersion of methylcholanthrene, whereas no tumor was found at the site of the mineral oil-methylcholanthrene injection. This indicates that the inducing agent in these two mice was the horse-serum dispersion of methylcholanthrene. The latent period of tumor induction was not accurately determined since a diagnosis of tumor was

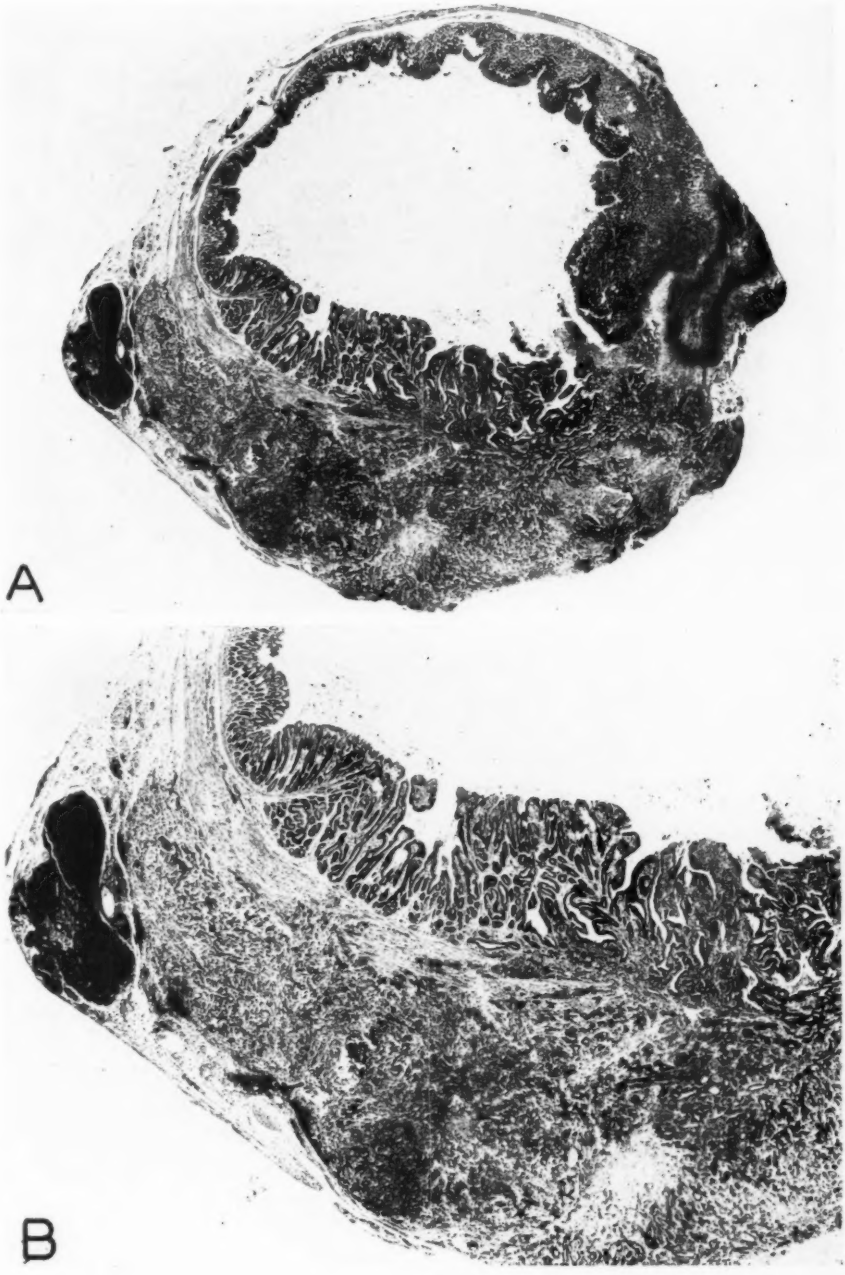
TABLE 2.—*Induced adenocarcinoma of pyloric stomach in mice*

Strain of mice	Sex	Age	Mice with tumors	Methylcholanthrene used	Vehicle	Survival of animals (in weeks)								Percentage of tumors	Tumors transplanted	
						19	22	23	33	37	38	39	46		Takes	Generations
		Weeks	*Number*	*Milligrams*											*Number*	*Number*
C3H	M	20-26	6	0.8	Serum	1	1	1	1	.....	.....	1	1	14	1	12
L	M	13-15	2	.14	Oil	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
				.6	Serum	.....	.....	.....	.....	1	1	.....	.....	50	.....	.....

not made until the autopsy. As determined by this method, the latent period ranged from 19 to 46 weeks, which indicated that, with the dose of 0.6 to 0.8 mg. of methylcholanthrene, the glandular mucosa of some C3H male mouse stomachs can be considered relatively susceptible to tumor induction.

The gross and microscopic appearance of induced adenocarcinoma of the stomach has been described and illustrated previously (*5*). In the present paper, a protocol is presented of a mouse that developed a gastric adenocarcinoma which has been successfully transplanted. This tumor has been designated in this Institute as mouse gastric adenocarcinoma No. 1. The tumor arose in a male strain C3H mouse which received an injection of 0.8 mg. of methylcholanthrene dispersed in horse serum 33 weeks before it was killed and autopsied (experiment 12, table 1). At autopsy there was a firm serosal nodule extending across the mid-anterior portion of the pyloric chamber of the stomach from the lesser to the greater curvature. The remainder of the pyloric chamber was firm and rigid. The mucosa opposite the serosal nodule was elevated, thick, and gray. Microscopic examination of the glandular stomach revealed an adenocarcinoma infiltrating all coats of the stomach and forming a large mass on the serosal surface (fig. 1). The growth extended from near the limiting ridge to the pylorus. On the mucosal surface of the tumor there were hyperplastic gland structures and an ulcer bordered by inflammatory and tumor cells and necrotic material. The mucous membrane on either side of the tumor showed atypical hyperplasia shading off into the adjacent mucosa. The cells of the tumor were arranged in small irregular acini chiefly and in solid nests and cords (fig. 2). The tumor cells were columnar, cuboidal, round, oval, and deeply hyperchromatic and rarely contained droplets of mucus. As many as four mitotic figures per high-power field were counted. The stroma was composed of relatively avascular adult connective tissue and was more abundant in the areas of the tumor where acini predominated. There were numerous areas of necrosis and inflammatory foci. The tumor has been transplanted subcutaneously into C3H mice for 12 generations and is still being carried in transplant. The first 6 generation transplants grew rapidly, reaching a diameter of 15 mm. in 2 weeks. The last 6 generation transplants have not grown so rapidly, requiring about 1 month to reach a diameter of 15 mm. The tumor continues to grow as an adenocarcinoma closely resembling

FIGURE 1.—*A*, Section through adenocarcinoma of glandular stomach. There are invasion and destruction of muscularis, the formation of a tumor nodule on the peritoneal surface, and an area of ulceration, × 12; *B*, Transition zone between the normal mucosa and the hyperplastic gland structure on the surface overlying the tumor. × 18.


A
B

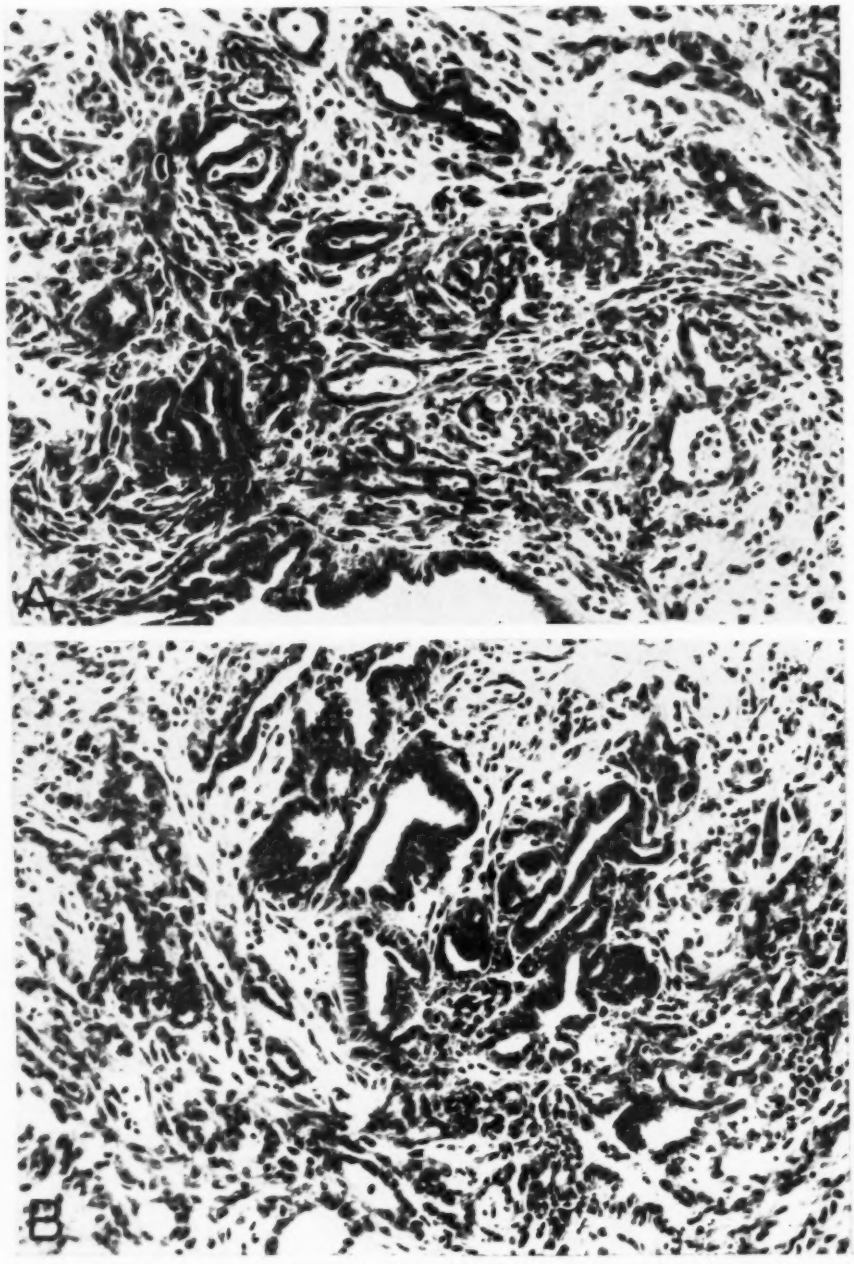


FIGURE 2, *A* and *B*.—Sections from the peritoneal nodule of adenocarcinoma shown in figure 1. × 195.

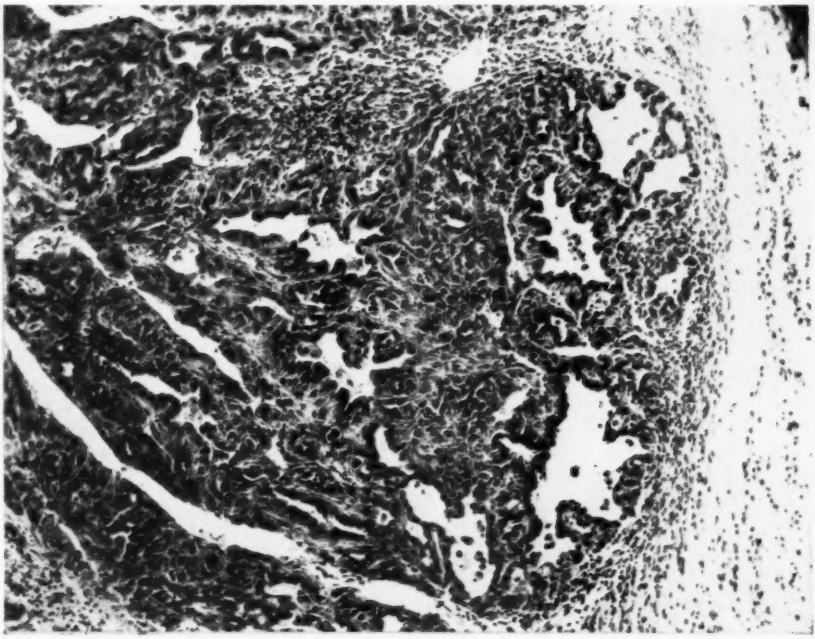


FIGURE 3.—Subcutaneous transplant of tumor shown in figures 1 and 2. This tumor is now in the twelfth generation transplant and still continues to reproduce the structural characteristics of the primary tumor. × 105.

the primary tumor (fig. 3), although in the last 2 passages the tumor has become more solid with fewer acini.

Two gastric adenocarcinomas occurred in strain I mice. This is interesting in view of the fact that mice of this strain spontaneously develop an adenomatous hyperplastic gastritis with mucosal glands sometimes displaced in the deeper layers of the gastric wall and in gastric blood vessels (*2*, *8*). These deep extensions of glandular fragments are always well-limited and not disorderly as in cancer, and there is frequently a suggestion of contact with the surface mucosa through a fold in the gastric wall. Although large irregular acini may be found indenting the basement membrane in several places, only one or two atypical gland fragments ever gain access to the deeper tissues in any given specimen. On the mucosal surface the lesion develops symmetrically, all specimens conforming within close limits to the same general pattern at any given age. With induced adenocarcinoma, there was a prominent tumor on the mucosal surface at the injection site. Microscopically, the atypical cells and acini of the gastric carcinoma infiltrated all coats of the gastric wall over a broad base forming a large neoplastic nodule on the peritoneal surface. The points just enumerated served to distinguish the spontaneous hypertrophic gastritis from the induced gastric carcinoma in the strain I mice.

ADENOMA

The data on induced adenoma of the pyloric stomach are presented in table 3. The first tumor was observed at autopsy

TABLE 3.—*Induced adenoma of pyloric stomach in mice*

Strain	Sex	Number of mice	Age	Hydrocarbon	Dose	Vehicle	Survival of mice (in months)														Mice with tumors	Involvement of muscularis
							3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
			Weeks		*Mg.*																		*Percent*	*Number*
A	M	2	9	Methylcholanthrene.	0.34	Oil								1		1							14	
A	F	1	9	do	.34	do															1		9	
C3H	M	2	20	do	.41	do			2														12	
C57 Black	M	3	20	do	.34	do		1				1										1	19	1
C	M	2	13	do	.34	do											1	1					14	
C3H	M	4	13	Dibenzanthracene.	.2	Lard						1		1					1		1		33	
C3H	M	7	20-26	Methylcholanthrene.	.8	Serum	1	3	2			1											17	2
C3H	M	5	22	do	.2	Oil	1	1					2				1						14	3
A	M	2	24-26	do	.14	do												1		1			7	1
A	M	2	30	do	.2	Serum					1	1											13	
I	M	4	13	do	.14	Oil					1			1	1		1						21	1
I	F	3	13	do	.14	do								1			2						33	2
C57 Black	F	4	12	do	.14	do			1						1	2							80	
Do	M	1	12	do	.14	do									1								14	
Dilute brown	M	1	17	do	.14	do			1														9	
Do	F	1	19	do	.14	do								1									50	

FIGURE 4.—Mucosal surface of posterior half of stomach showing forestomach to right, limiting ridge and pyloric chamber to left. The esophagus is above, and the duodenum is in upper left corner. In the distal portion of the pyloric chamber is a broad flat nodule 4 mm. in diameter with a depressed center and slightly elevated, rolled margin. This nodule was visible through the serosa. Microscopic section of this lesion revealed that it extended down to the muscularis but did not penetrate it, hence the lesion is not classified as malignant, although grossly suggestive. Photograph of gross specimen enlarged.

12 weeks, and the last tumor 69 weeks after injection. Of the 44 adenomas observed, 10 showed superficial infiltration of the muscularis of the stomach.

The adenomas were usually grossly detectable at autopsy. When small, they consisted of varying degrees of thickening or folding of the mucosa. It was frequently possible to identify the injected methylcholanthrene grossly in the wall of the stomach, and the mucosal lesion was virtually always at the injection site. In some specimens of stomach there was a firm, well-circumscribed area with a depressed, punched-out center 2 to 4 mm. in diameter surrounded by an elevated ruffled border of mucosal thickening which shaded off gradually into the surrounding mucous membrane (fig. 4). A few polypoid lesions occurred, the largest being 3 mm. in diameter.

Microscopically, the lesions classified as adenoma varied in extent from small nodules of atypical acini in the mucosa and submucosa to large plaquelike glandular masses occupying the submucosa and invading the superficial muscularis (figs. 5 and 6). The glands composing the adenomas were irregular and distorted and

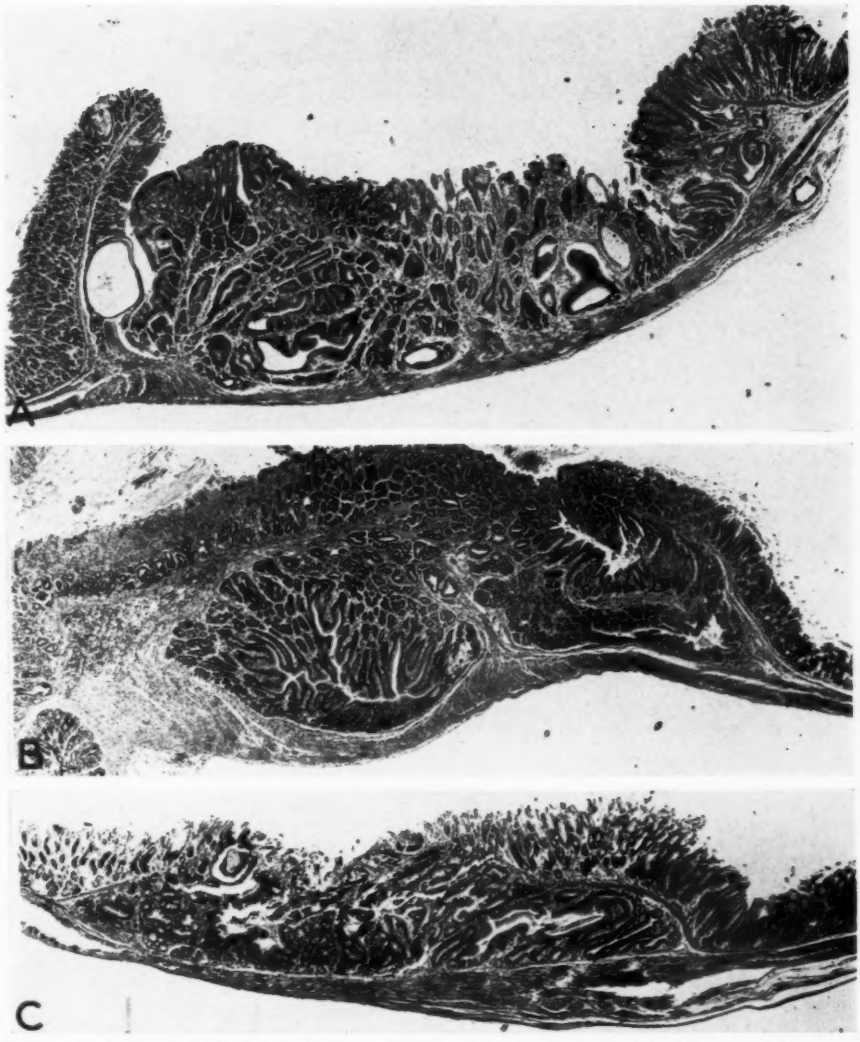


FIGURE 5.—Adenomas of the stomach from three mice. *A*, Lesion is markedly atypical, and except for the fact that it has not infiltrated through the muscularis to the peritoneum, it is structurally indistinguishable from other lesions which had permeated the gastric wall and were classified as adenocarcinoma. × 27; *B*, Lesion is better differentiated than in *A*, but it has extended down to the muscularis. × 27; *C*, Muscularis mucosae is destroyed, and the submucosal nodule is composed of atypical glands which rest on the muscularis. × 39.

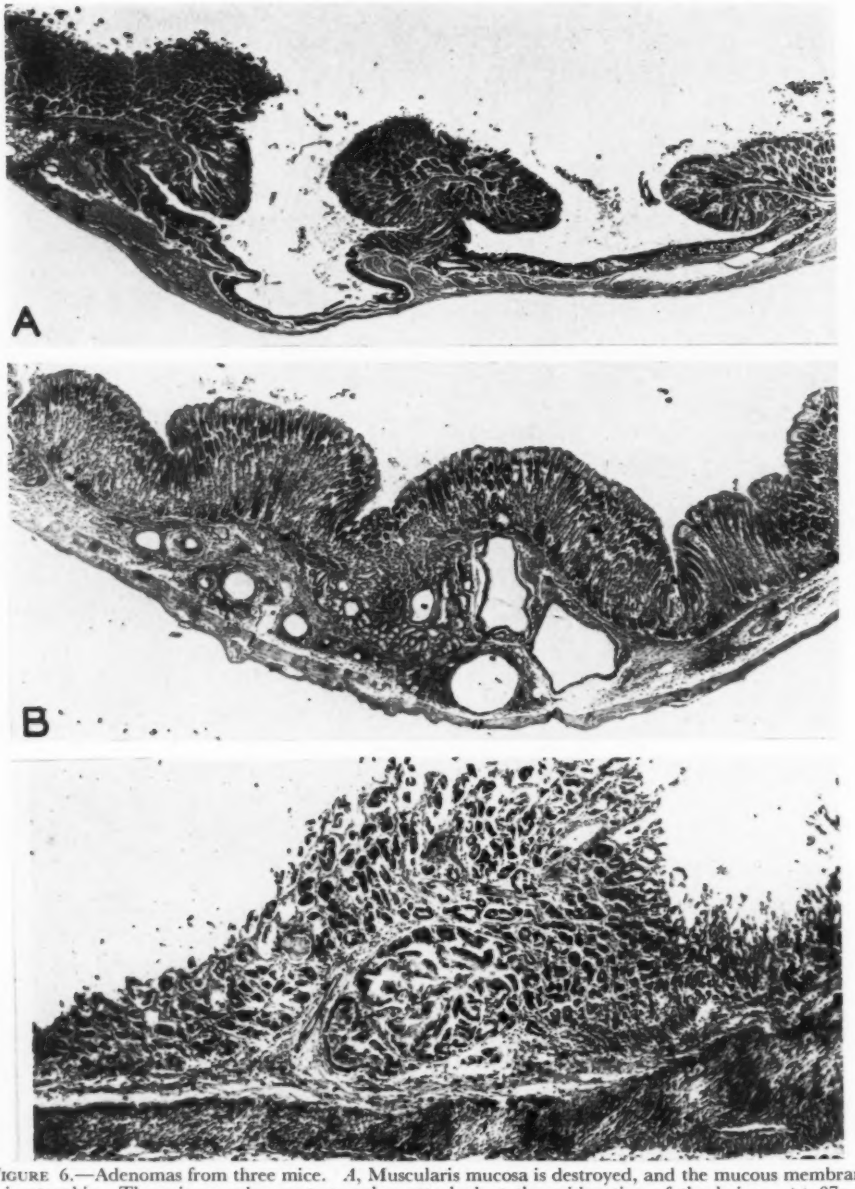


FIGURE 6.—Adenomas from three mice. *A*, Muscularis mucosa is destroyed, and the mucous membrane is atrophic. There is an adenomatous polyp attached at the midportion of the lesion. × 87; *B*, Injection site is represented by vacuolated spaces lying in an inflammatory area in the submucosa to the left of the center. To the right of this, the muscularis is destroyed and the submucosa contains a nodule of atypical dilated glands. In this area the superficial portion of the mucosa is unchanged. × 30; *C*, Small focus of atypical glands in the base of the mucosa. This is the earliest change that has been observed. × 80.

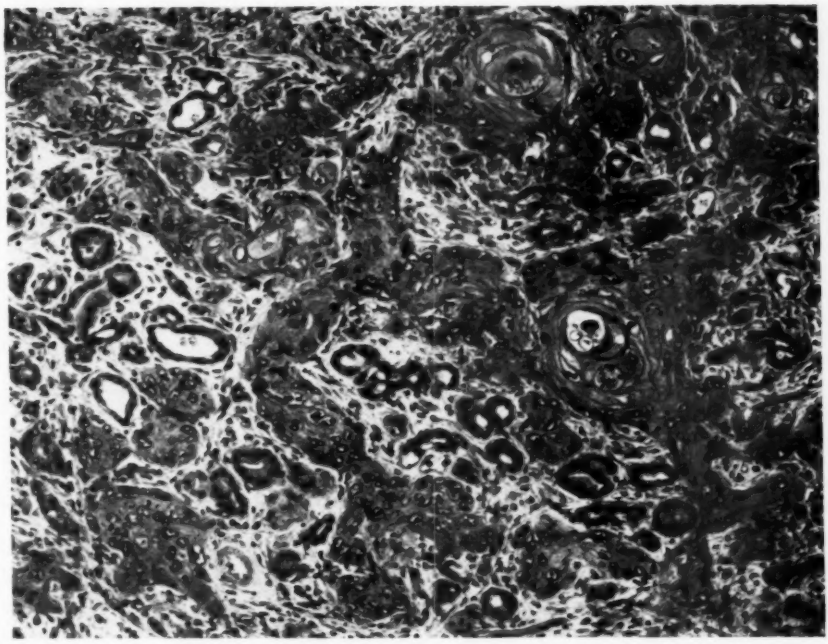


FIGURE 7.—Adenoacanthoma of stomach, metastatic in liver. The glandular and squamous elements are readily recognized. × 153.

lined by hyperchromatic, cuboidal, and columnar cells which varied in size, shape, and staining, and sometimes contained mucin. The earliest change appeared in the basal half of the mucous membrane. The glands in the upper half of the mucosa frequently showed little or no abnormality even in the presence of a relatively large adenomatous nodule in the basal half of the mucosa and the submucosa. The explanation of this is not entirely clear. It may be that since the deeper glands of the mucosa were in closer contact with the injected methylcholanthrene they were more frequently and more markedly affected. On the other hand the difference in the neoplastic reaction of the cells at these two levels of the glandular mucosa may be more fundamental. Lorenz and Stewart[3] have fed methylcholanthrene and dibenzanthracene in aqueous oil emulsions to a large number of mice for varying periods of time, over a year in some instances. Some mice ingested as much as 250 mg. of the carcinogen. The glandular mucosa of these mice was completely refractory to tumor induction despite the fact that many of them developed squamous-cell carcinoma of the forestomach and adenocarcinoma of the small intestine. This indicates, therefore, that there is some powerful mechanism which protects the glandular mucosa of the stomach from the carcinogenic influence of ingested carcinogenic hydrocarbons. However, this mechanism is apparently not operative when carcinogen is injected into the wall of the stomach. But even under these circumstances, it appears that the surface of the mucous membrane is not affected whereas the

[3] *Unpublished data.*

basal half may develop adenocarcinoma.

The majority of the gastric adenomas were depressed below the surface, sometimes eroded, but usually not ulcerated; the margins were elevated; and the muscularis mucosae were destroyed. Three were polypoid. In one specimen the base of an ulcer was lined by atypical glands which fanned out to form a large bottle-shaped nodule occupying the submucous space and extending down to the muscularis. In a few cases atrophy of the mucosa was in association with the neoplasm (fig. 6, *A*). There were usually slight inflammatory changes about the encysted methylcholanthrene, but the induced adenomas were ordinarily relatively free from inflammatory change.

Adenoacanthoma

There were eight adenoacanthomas of the stomach. Four of the tumors involved both the forestomach and pyloric stomach so that the source of the squamous and glandular elements composing the tumor can be readily explained. In the other four mice the neoplasm was confined to the pyloric chamber, hence the only explanation for both squamous and glandular elements in the tumor is that the glandular mucosa may, under the carcinogenic influence of methylcholanthrene, differentiate into both glandular and squamous neoplastic elements. All the adenoacanthomas were situated in part in the area of the stomach injected with methylcholanthrene, and the bulk of the tumor was confined to the pyloric chamber. The tumors measured from 6 to 10 mm. in diameter and were elevated on the mucosal surface, causing varying degrees of obstruction to the pyloric chamber. Nodular masses were present on the serosa opposite the tumor, and the stomach was frequently adherent to the liver, pancreas, and spleen. Microscopically, the tumors were composed of basal cells, prickle cells, and flattened squamous cells arranged in anastomosing cords which showed pearl formation and keratin. Intermingled with these were irregular atypical gland structures lined by cuboidal and columnar cells which varied in size, shape, and staining. One of the tumors had large microcysts which individually were lined by both squamous and columnar cells. The stroma was composed of moderately vascular, adult, connective tissue infiltrated with inflammatory cells in areas. One of the tumors yielded a metastases in the liver, reproducing adenoacanthoma (fig. 7).

Sarcoma

When sarcoma developed, it appeared relatively early, grew rapidly, attained a large size, formed large nodules on the gastric serosa, infiltrated adjacent structures, and metastasized frequently. The tumors varied from 7 to 25 mm. in diameter. They formed adhesions to the spleen, pancreas, liver, intestines, and omentum in several cases. Eleven of the mice with gastric sarcoma showed metastases or extragastric extension of the tumor. One of the tumors was transplanted for two generations after which it was discontinued. The majority of the sarcomas were confined to the pyloric chamber but in a few instances also involved the wall of the forestomach. One sarcoma was confined to the wall of the forestomach. As a rule the mucous membrane overlying a sarcoma remained intact and could be identified as a distinct, thin, gray line after the fixed gross specimen was sectioned. The lumen of the stomach was more or less completely obstructed by submucosal pressure of the tumor. The cut surface of the tumor was firm and gray, with areas of hemorrhage and necrosis. Microscopically, the sarcomas were composed of rapidly proliferating spindle-shaped cells

arranged in interlacing bundles with a characteristic sarcomalike distribution of reticulum but not much collagen. Several of the tumors were stained by the phosphotungstic acid-haematoxylin stain, and the tumor cells showed fibroglia fibrils which allowed a classification of fibrosarcoma. The cells in the majority of the tumors were hyperchromatic and fairly uniform in size although some tumors showed marked variation in cellular size, shape, and staining, with numerous giant cells. Most of the tumors were abundantly supplied with thin-walled vessels, and there were scattered areas of necrosis, hemorrhage, and inflammatory foci.

Mixed Tumors

There was a total of 20 mixed epithelial and connective-tissue tumors of the stomach either mixed adenocarcinoma and sarcoma or mixed adenoacanthoma and sarcoma. Of the 2 tumors of mixed adenoacanthoma and sarcoma, 1 involved both the forestomach and pyloric chamber while the other was confined to the pyloric chamber. The majority of the mixed adenocarcinoma and sarcoma were confined to the pyloric chamber. These tumors, like the unmixed sarcomas, developed early, grew rapidly, and attained a large size. The largest tumor observed in the entire series was a mixed adenocarcinoma and sarcoma which measured 35 x 30 x 30 mm. As stated previously, the unmixed sarcomas tended not to infiltrate the gastric mucosa so that grossly the mucosal line could be observed intact in the majority of cases. This was not true of the mixed tumors, which as a rule formed elevated nodules on the mucosal surface. Otherwise these mixed tumors were not distinguishable grossly from the sarcomas. Microscopically, in the majority of the mixed tumors the epithelial elements were most numerous in that portion of the tumor closest to the mucosal surface. In the specimens in which the epithelial glandular elements were more widely scattered throughout the tumor, those most distant from the mucous surface of the stomach showed cystic and regressive changes. This suggests that the rapidly proliferating sarcoma was outgrowing the epithelial elements. The precise origin of the sarcoma cells was not clear in most cases, but in a few early tumors there appeared to be a pronounced proliferation of the connective tissue stroma of the mucous membrane. The mixed epithelial and connective tissue tumors were equally as malignant as the sarcomas since of the 20 tumors infiltration or metastases occurred in 7.

SUMMARY

The carcinogenic hydrocarbons, 20-methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene, were introduced into the wall of the glandular chamber of the stomach of mice of both sexes and of strains C3H, A, C, C57 Black, I, and dilute brown. The dose of carcinogen varied from 0.14 to 0.8 mg. of hydrocarbon. Four vehicles were employed as follows: (1) A solution in mineral oil containing 6.8 mg. of methylcholanthrene per cubic centimeter; (2) a solution in lard containing 0.4 mg. of 1, 2, 5, 6-dibenzanthracene per cubic centimeter; (3) a dispersion containing 20 mg. of methylcholanthrene per cubic centimeter in horse serum; and (4) cotton thread impregnated with approximately 0.5 mg. of methylcholanthrene, benzpyrene, or dibenzanthracene and stitched directly into the wall of the glandular stomach. The solutions and dispersions were injected into the wall of the glandular stomach by means of a tuberculin syringe and a 27-gage hypodermic needle.

A total of 293 mice was used. At autopsy the following tumors of the stom-

ach were found: involving the glandular stomach, adenoma 44, adenocarcinoma 8, adenoacanthoma 8, mixed adenocarcinoma and sarcoma 18, mixed adenoacanthoma and sarcoma 2, and sarcoma 30; in the forestomach, squamous papilloma 44, and squamous carcinoma 14.

In all the experiments except those with the impregnated cotton thread, some type of glandular tumor either in pure form or mixed with sarcoma or squamous elements was induced. In the experiment with the impregnated cotton threads 19 mice were employed, but only 1 tumor, a sarcoma, was observed at autopsy.

Adenocarcinoma of the glandular stomach was induced in six male strain C3H mice which received 0.8 mg. of methylcholanthrene dispersed in horse serum and two male strain I mice which received 0.6 mg. of methylcholanthrene in the same vehicle and in addition 0.14 mg. of methylcholanthrene in mineral oil. The interval between the time of injection and the finding of the gastric neoplasm at autopsy in these eight mice was as follows: 19, 22, 23, 33, 37, 38, 39, and 46 weeks. In all the mice the tumor extended through the muscularis of the wall of the stomach and infiltrated the peritoneum. Extragastric extension and metastases were not observed. One adenocarcinoma, transplanted subcutaneously into mice of the same strain, is now in the twelfth generation and continues to reproduce the structural characteristics of the original growth. This transplant reaches a diameter of 15 mm. in from 2 to 4 weeks.

With regard to the other malignant tumors, extragastric extension and metastases to the liver, pancreas, mesentery, or peritoneum occurred as follows: adenoacanthoma, 1; sarcoma, 11; mixed adenocarcinoma and sarcoma, 6; mixed adenoacanthoma and sarcoma, 1.

Four adenoacanthomas involved both the glandular stomach and the forestomach so that their origin from both squamous and glandular mucosa is probable. The other four tumors were confined to the glandular stomach, hence the only explanation for their origin would be the glandular mucous membrane.

ADDENDUM

Since this paper was prepared for publication, adenocarcinoma of the glandular stomach has been found at autopsy in three more male mice of strains C3H, I, and C57 Brown which received, respectively, 0.4 mg. of 20-methylcholanthrene dispersed in horse serum, 0.6 mg. of the same dispersion, and 0.14 mg. of 20-methylcholanthrene in mineral oil; the mice died at 13, 26, and 51 weeks, respectively, following injection. The strain C57 Brown mouse was the first animal to develop the tumor when mineral oil was used as the vehicle. This brings the total number of induced gastric adenocarcinomas to 11.

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A Spontaneous Fibrosarcoma of the Foreleg and Paw in a C3H Mouse

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We have had opportunity to study, in both the primary and transplanted states, a fibrosarcoma involving the foreleg and paw of a C3H mouse. Since spontaneous fibrosarcoma in this species is relatively uncommon, it seems desirable to place this case on record (*1*). The tumor is designated in our laboratory as fibrosarcoma 4801.

HISTORY OF TUMOR

The tumor in the right foreleg and paw appeared in a 9-months-old virgin female C3H mouse of the Andervont line. The mouse had been nursed by its own mother and at weaning was placed on a high cystine-low protein synthetic diet. The animal developed and gained weight normally. When the mouse was 8 months of age, a hard swelling was noticed in the soft tissues of the right foreleg. One month later this measured 1 cm. in diameter and involved the paw as well as the lower leg. A roentgenogram taken at this time showed a soft tissue swelling in the right lower leg and paw. The cortex of the distal end of the ulna showed some roughening laterally, and opposite this, well out in the soft tissue shadow, were crescent-shaped areas of considerably increased density (fig. 1). The animal was killed, and at the time of autopsy small pieces of tumor were transplanted by the trocar technique to the subcutaneous tissues of five mice belonging to the homologous strain and line. In all five animals takes were palpable within 3 weeks. The tumor has continued to be readily transplantable and is now in the fourth generation.

GROSS PATHOLOGY

The primary tumor appeared as a somewhat globular mass measuring 1.0 cm. in diameter, involving the anterior, lateral, and medial aspects of the right lower leg and paw. It was covered by intact reddened skin. Cut sections of tumor revealed gray, firm or gelatinous tissue with gritty foci suggesting bone. The tumor appeared to be attached to the ulna. No metastases were observed, and the internal organs were negative.

The transplants were firm and often covered by reddened skin. Cut sections of these revealed firm, pearly gray tissue but no areas suggesting bone. No me-

FIGURE 1.—Enlarged roentgenogram of right foreleg. There is a large soft tissue mass which contains increased areas of density. The lower end of the ulna is roughened.

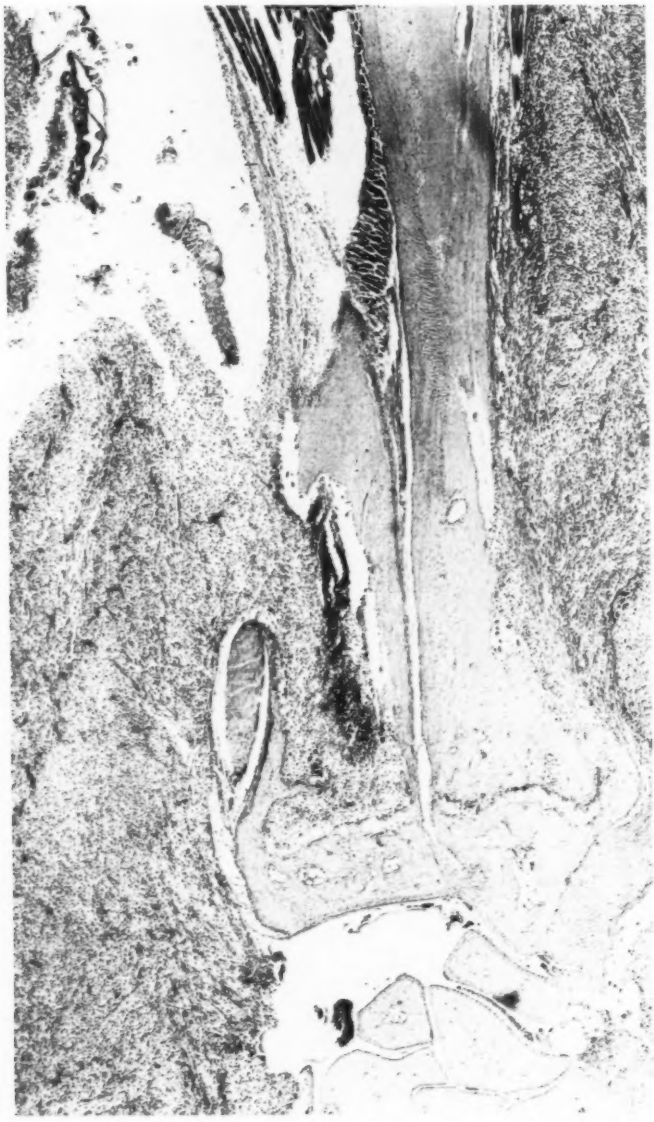

FIGURE 2.—Right lower leg and paw of C3H mouse. Tumor surrounds the lower leg and ankle. The cortex of the radius is partially eroded. The cortex of the ulna shows one large defect involved by tumor. The medullary cavity of the ulna is partially replaced by tumor. ×40.

tastases have been observed from transplanted tumors in mice coming to autopsy.

MICROSCOPIC PATHOLOGY

The primary tumor was invasive, having partly enveloped and eroded the cortex of the radius and ulna as well as several of the carpal bones (figs. 2 and 3). The involvement of bone was most extensive in the radius where one side of the cortex was completely penetrated, and the tumor involved the medullary cavity. It is of interest and importance with respect to determining the site of origin of the tumor that while the tumor had involved the medullary cavity, it had not completely destroyed the bone marrow (fig. 4).

The primary tumor and the transplant were similar microscopically except for the presence of bone in the primary tumor. Both were composed of elongated cells which showed a marked tendency to form interlacing bundles (fig. 5). The cytoplasm of the tumor cells stained faintly with eosin. The nuclei were elongated, their chromatin was distributed in the form of finely divided particles, and no definite nucleoli were present. Mitotic figures were found in moderate numbers. Fibroglia fibrils associated with the individual tumor cells were demonstrated with Mallory's phosphotungstic acid-haematoxylin stain (fig. 6, *A* and *B*). With stains for reticulum an abundance of this substance was observed, and it was intimately associated with the individual cells (fig. 6, *C*). Within the primary tumor were several spicules of bone.

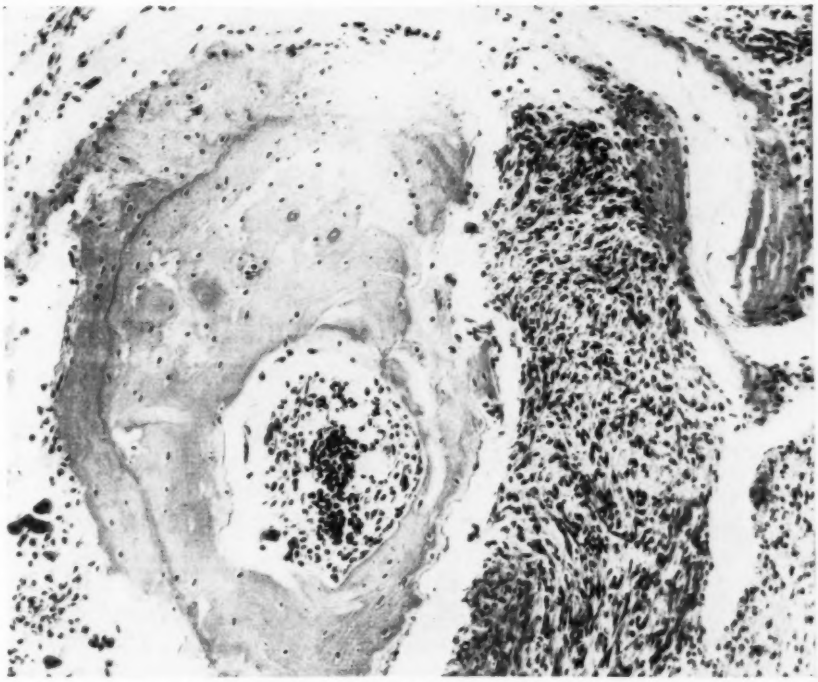

FIGURE 3.—The cortex of a carpal bone is eroded by tumor. The marrow of the bone is unaffected. ×120.

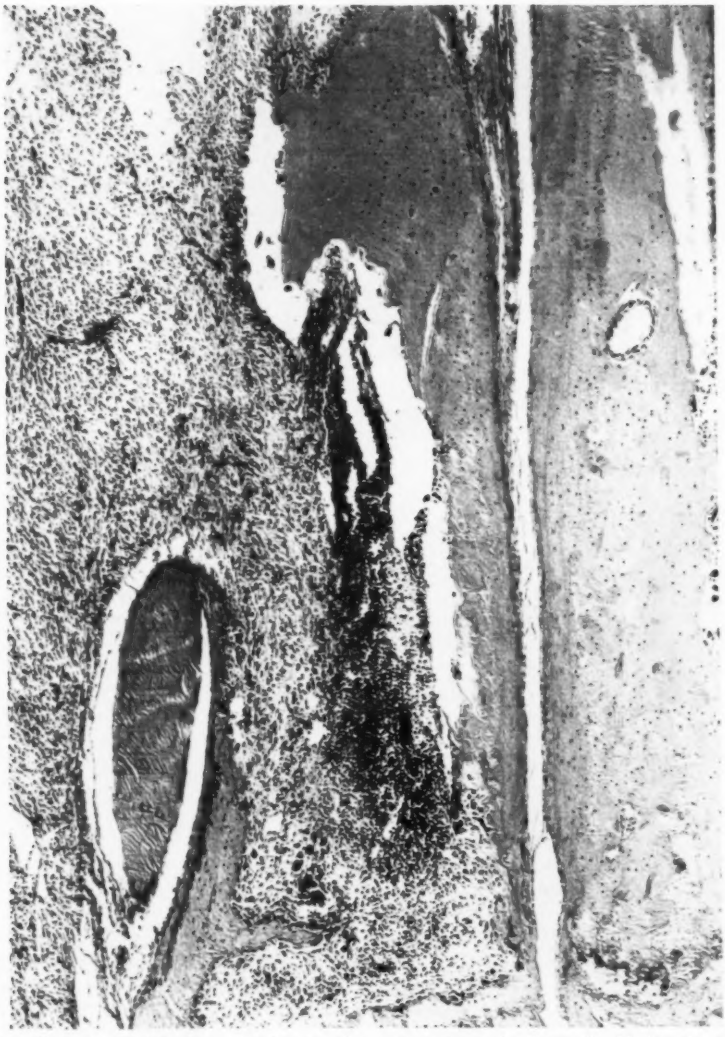

FIGURE 4.—Higher magnification of defect in ulna cortex shown in figure 2. Notice the incomplete replacement of the medullary cavity by tumor. × 90.

These were well-differentiated and showed no mitotic activity, atypical cells, or transitions from the tumor tissue (fig. 7).

Studies for alkaline phosphatase by the staining technique of Gomori (*2*) were carried out on transplant material. None of this enzyme was demonstrated in the tumor cells by the use of this technique.

The Golgi material of the tumor cells when compared with that of the normal fibroblast appeared to be reduced slightly in amount. The Golgi apparatus was similar in form to that of the normal cell and was regularly juxtanuclear, more

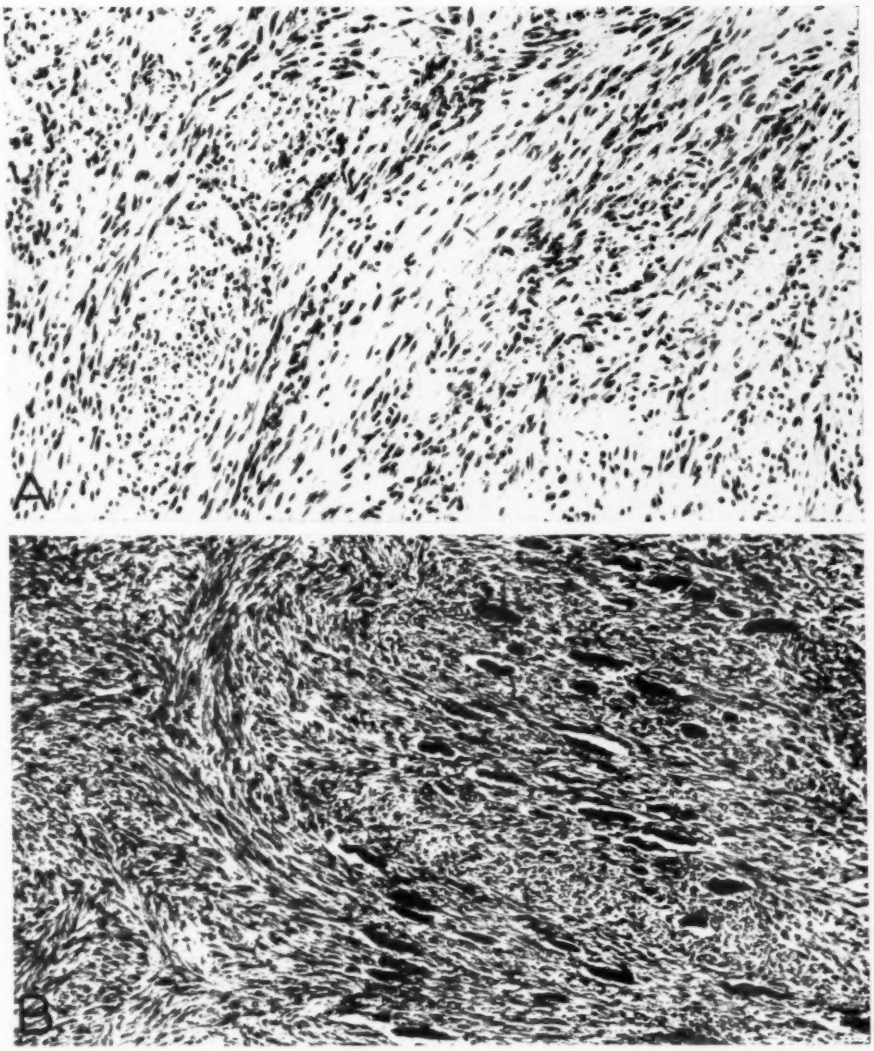

FIGURE 5.—*A*, Primary tumor showing interlacing bundles of elongated cells. ×150. *B*, Third-generation transplant. Notice fragments of invaded skeletal muscle in tumor. Phosphotungstic acid-haematoxylin stain. ×130.

frequently toward one pole of the nucleus, less frequently at its lateral margin. The mitochondria were similar to those of the normal fibroblast. They were mostly filamentous and tended to be oriented in general along the long axis of the cell.

DISCUSSION

That the tumor under consideration is of fibroblastic origin is supported by the

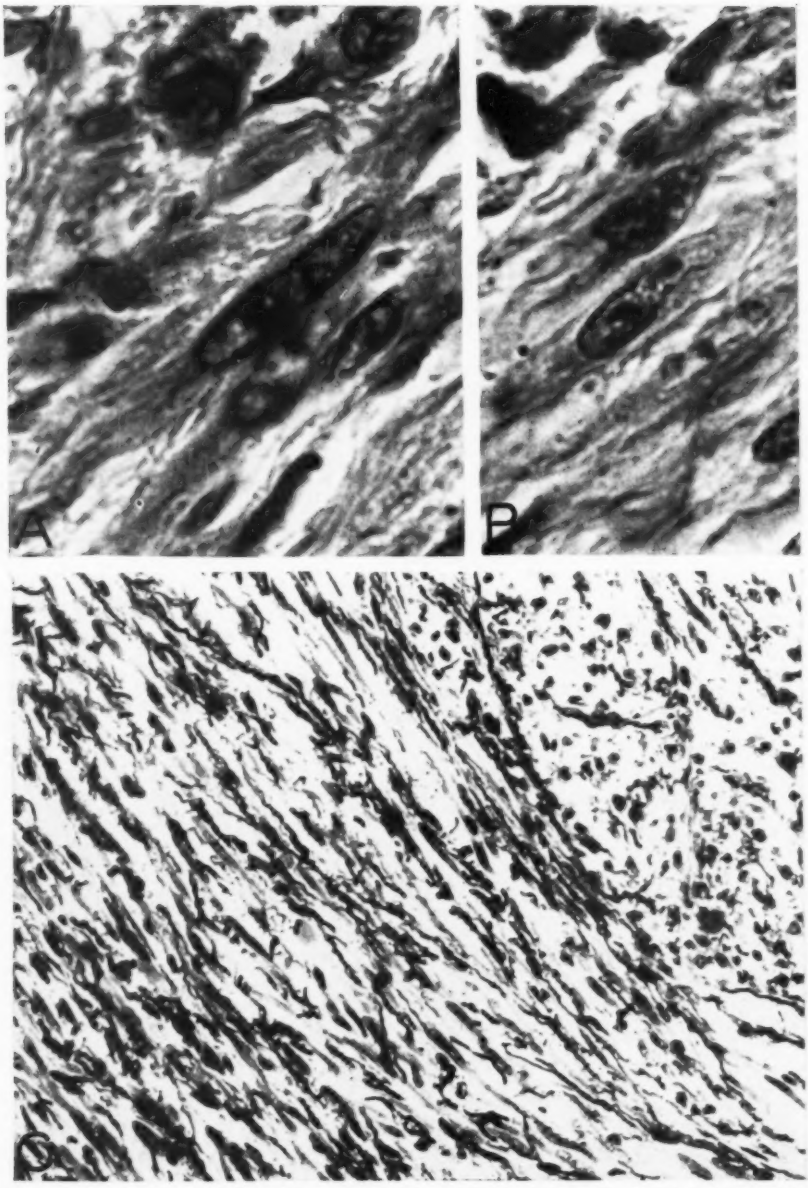

FIGURE 6.—*A* and *B*, Third-generation transplant of fibrosarcoma showing fibroglia fibrils. Phosphotungstic acid-haematoxylin stain. ×1760; *C*, Primary tumor. Reticulum fibers intimately associated with individual tumor cells. Laidlaw's stain for reticulum. ×300.

shape of the cell, the tendency for the cells to form interlacing bundles, the intimate association of the individual cells with reticulum (*3*), and, of utmost importance, the presence of fibroglial fibrils (*4, pp. 278–288*). That the tumor is malignant is evidenced by its active growth and by its invasive and destructive characteristics.

Whether the tumor arose in bone or in soft tissue requires some discussion. Two possibilities present themselves: (1) That the tumor arose within the medullary cavity of the bone and so should be classed as an intramedullary fibrosarcoma of bone; or (2) that it originated in periosteal or other extraosseous connective tissue. Before considering this, the presence of bone within the primary tumor should be explained. We believe that this bone does not represent a true part of the tumor, for although it lay in a malignant tumor it showed neither characteristics of malignancy nor any transition from the tumor tissue. Further, none of the transplants contained any such elements. It seems reasonable to explain the presence of bone in the primary tumor on the basis that as the tumor attacked the cortex of the ulna, several spicules of bone became detached from the shaft and were progressively separated from it by the interposed enlarging tumor. The absence of demonstrable alkaline phosphatase in the tumor by the staining technique indicates that if any of this enzyme was indeed present it must have existed in amounts less than would be expected in a tumor with bone-forming characteristics. Neoplastic tissue which has the capacity to form bone has been shown to have considerable alkaline

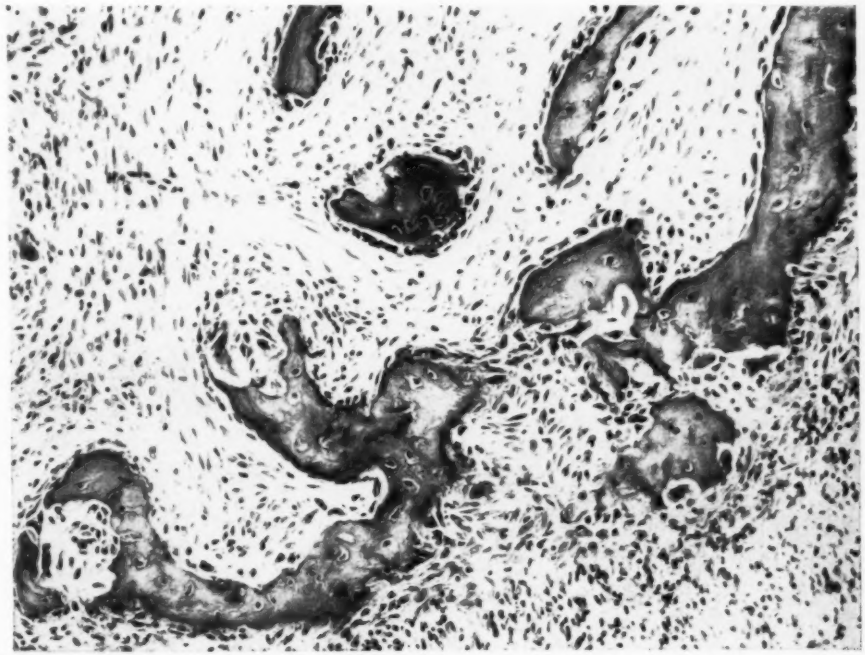

FIGURE 7.—Bone in primary tumor. Notice adult state of bone, absence of mitosis, and lack of transitional stages between it and the tumor in which it lies. ×180.

phosphatase, easily demonstrable by the staining method (*5*).

The absence of extensive replacement of bone marrow in the medulla of the radius seems to contradict the possibility that the tumor arose within the medulla. One would expect more extensive involvement of the medullary cavity had the tumor been intramedullary in origin. We offer this case, then, as a fibrosarcoma arising in the soft tissues. Whether it arose in the periosteum or from other extraosseous connective tissue is impossible to determine in a tumor as extensive as this one.

SUMMARY

The history and pathology of a spontaneous transplantable fibrosarcoma involving the foreleg and paw of a virgin female C3H mouse are reported.

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A Review of Some Spontaneous Neoplasms in Mice[1]

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From the time of the earliest studies in experimental cancer to the present an abundance of evidence has been presented which indicates clearly the similarity between neoplasms of mouse and man. A number of authors have reported on the pathology of large collections of common and rare spontaneous (*1–13*) and induced (*14–21*) tumors in mice. In the present paper, we report some of our observations at the National Cancer Institute on the pathology of some spontaneous tumors in the mouse. While several relatively uncommon tumors are mentioned, emphasis is placed on the more common varieties of spontaneous neoplasms, such as those arising in the lung, mammary gland, liver, and in the region of the salivary gland.

MAMMARY TUMORS

The spontaneous mammary tumors from inbred strains of mice form one of the most useful groups of neoplasms for certain investigations in the field of cancer research. The C3H strain of mouse is typical of one of the high-incidence strains produced through brother-and-sister mating. The representative neoplasms from this strain are shown in the illustrations in this review.

The mammae of the mouse usually consist of 5 pairs of glands, and multiple tumors frequently arise in them (fig. 1, *C*). Of 250 spontaneous tumors in 135 female C3H mice, 91 percent were found on the ventral surface and the remaining 9 percent on the dorsal expansions of the mammary glands. Tumors appear more commonly in the anterior than in the posterior mammae. In virgin females these tumors are usually observed at about 10½ months and in breeding females at about 8½ months (*22*). The average duration of life after the tumor is first observed is 6 to 7 weeks.

Grossly, the smallest tumors appear as pea-sized, firm, white nodules containing minute, taut, cystic formations. As the tumor increases in size, hemorrhagic fluid fills these expanding cysts. Frequently they rupture, and the tumors also become softened by necrosis. They may grow rapidly to attain a large size approximating or even exceeding the weight of the host animal.

Apolant (*1*) has classified the mouse breast tumors which he studied as follows: adenoma, cystadenoma, adenoma malignum, papillary cystadenocarcinoma, cystadenocarcinoma, and adenocarcinoma. In our material no benign adenoma or cystadenoma was observed even in the smallest tumor recognized macroscopically. Adenocarcinoma (fig. 1, *A*) and cystadenocarcinoma (fig. 1, *B*) comprised nearly all of the tumors. In the former, areas of carcinoma solidum were common, and in the latter, papillary projections (fig. 1, *D*). One uncommon type was the adenosquamous carcinoma (fig. 1, *E*).

Microscopically, the adenocarcinoma is

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composed primarily of small, well-formed acini lined by a single layer of cuboidal cells with ovoid, lightly chromatic nuclei and scant cytoplasm. Distinct lumina are apparent where the individual acini are in loose apposition but are partially obscured where acini are closely packed together. Frequently no acinar structure can be made out, and instead there are solid masses of hyperchromatic epithelial cells containing many mitotic figures. Interstitial connective tissue stroma is scant as are the slender capillaries and compressed sinusoids. Such an adenocarcinoma invariably contains small or even large cysts. When cysts are abundant, the tumor is designated as a cystadenocarcinoma. In these cystic structures spurs of hyperplastic epithelium and papillary projections are often found. Occasionally they invade the basement membrane of the cyst. In the rare adenosquamous carcinoma, in addition to acinar structures squamous cells and keratinizing epithelial pearls form a part of the tumor.

HEPATIC TUMORS

The spontaneous hepatoma occurs with varying frequency, being highest in the C3H strain among the several strains studied at the National Cancer Institute. In the local colony of this strain Andervont (*22*) found an incidence of 5.7 percent of spontaneous hepatomas in 157 females from 11 to 19 months of age, while in 341 males of the same age group, the incidence was 25.8 percent. The incidence varies with age, as evidenced by the fact that at 11 months 10 percent of the males show the spontaneous hepatoma, and older animals show a progressively higher incidence of tumor. As is the case with other strains, the lesion in strain C3H mice is hardly ever observed in mice under 11 months of age. Spontaneous hepatoma is distinctly less common in strains A, Y, and C than in the C3H (*23*). In a group of 800 strain A mice of both sexes varying from 4 to 16 months of age the incidence was less than 1 percent. One hundred Y male and female mice from 10 to 16 months of age yielded 2 hepatomas, an incidence of 1.7 percent. None were found in 150 strain C females between the ages of 10 and 24 months (*24*).

The spontaneous hepatoma is practically always a solitary lesion appearing as a gray or grayish-yellow, soft, friable mass covered by an intact hepatic capsule (*24, 25, 26*). The mass may reach a diameter of 2.0 cm. and is occasionally pedunculated, being attached to the liver by a strand of attenuated hepatic tissue. The livers bearing the tumors show neither gross nor microscopic evidence of cirrhosis.

While metastases of hepatoma have been reported (*10, 27*), we have seen none in a fairly large number of animals examined. We are carrying by transplantation two spontaneous hepatomas, one arising in a C3H mouse and one in a strain A animal (*26*) (fig. 1, *G*).

Microscopically, the primary spontaneous hepatomas have rounded, nonencapsulated margins which encompass the adjacent hepatic tissue. The tumors are composed of cells resembling hepatic parenchymal cells which alternate with endothelial-lined blood sinuses (fig. 1, *F* and *G*). A delicate fiber of reticulum occasionally separates the cell from the sinus, while the cells of the tumor resemble very closely those of the normal liver. There is lack of a true lobular pattern as seen in the liver. Bile ducts, whenever found in the tumors, do not assume a constant position with respect to the cords of tumor cells and usually lie toward the periphery of the tumor mass. These facts, together with the absence of bile ducts in transplants of spontaneous hepatomas, support the idea

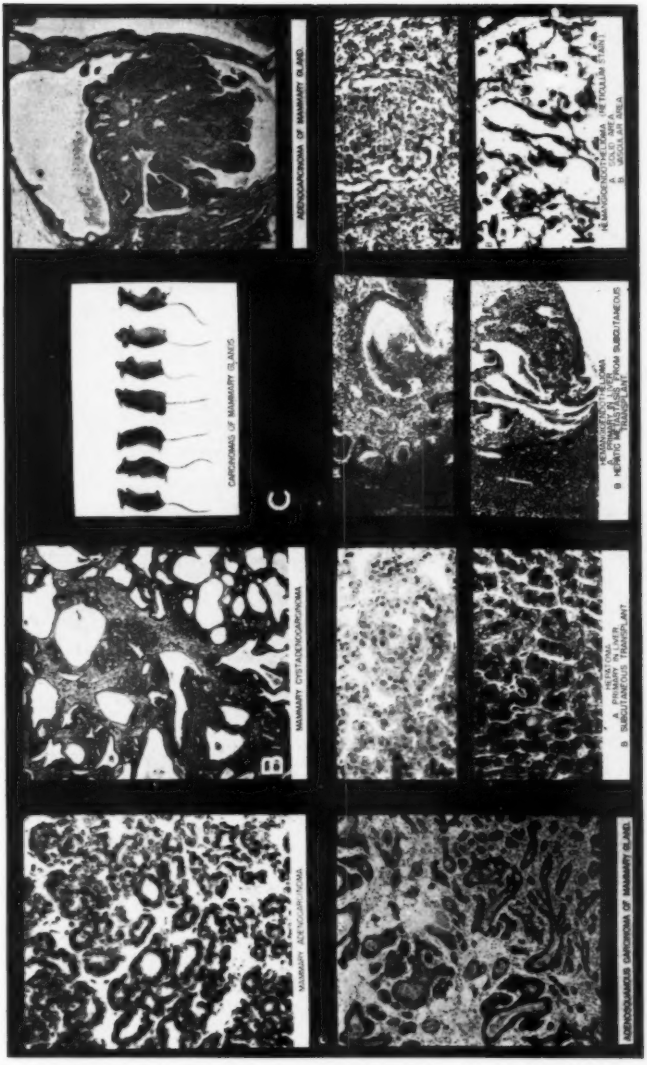
MAMMARY ADENOCARCINOMA
MAMMARY CYSTADENOCARCINOMA
CARCINOMAS OF MAMMARY GLANDS
ADENOCARCINOMA OF MAMMARY GLAND
B
C
A. PRIMARY IN LIVER
B. SUBCUTANEOUS TRANSPLANT
B. HOMOTRANSPLANT FROM SUBCUTANEOUS TRANSPLANT
A. SOLID TYPE
B. SCIRRHOUS AREA
ADENOCARCINOMATOUS MYOMA OF UTERUS
ADENOCARCINOMATOUS MYOMA
ADENOCARCINOMATOUS MYOMA OF UTERUS
ADENOCARCINOMA OF MAMMARY GLAND

FIGURE 1.

that the bile ducts are mere inclusions of preformed elements.

The tumor cells, while fundamentally resembling those of the hepatic parenchyma, exhibit some differences, namely, greater size and faintly basophilic cytoplasm. With experience, these characteristics serve as a valuable guide in distinguishing liver from tumor tissue.

Dalton and his coworkers have shown that in the tumor cells of both primary and transplanted spontaneous hepatomas the Golgi apparatus on the one hand differs strikingly from that of normal parenchymal hepatomas in the mouse (*28*) and the rat (*29*). In the normal hepatic cell the Golgi apparatus is distributed along the periphery of the cell near that margin forming part of the bile canaliculus while in the hepatoma the Golgi apparatus is a hypertrophied network removed from the cell membrane and occupies a juxtanuclear position.

We have not identified any bile pigment in the spontaneous hepatoma, although we have observed, as have others (*30*), peculiar hyalin bodies in both primary and transplanted growths which may possibly represent inspissated bile. These bodies usually lie within the tumor cell cytoplasm and occasionally in the intercellular spaces. They are globular and about equal in size to a tumor cell nucleus. With the haematoxylin and eosin stain they are fairly homogeneous, rarely basophilic, occasionally pale green, and most commonly eosinophilic. Mallory's aniline blue stain brings out three zones: a central blue, an intermediate unstained area, and a margin of blue.

A number of transplants have been studied for fat, glycogen, and alkaline phosphatase by the sudan IV, Best's carmine, and Gomori's method (*31*) for alkaline phosphatase, respectively, with the following results (*26*): Fat and glycogen are very abundant in the cytoplasm of the tumor cells, very often present in apparently greater amounts than that observed in the livers of the mice carrying the transplants studied. Alkaline phosphatase is not indentifiable by the staining method in the cytoplasm of the tumor cells.

It is of interest that, except for the usual multiplicity of the induced hepatoma as contrasted with the solitary character of the spontaneous hepatoma, the two have much in common microscopically. The general histologic picture is very similar, the Golgi apparatus indistinguishable, and the studies for fat, glycogen, and alkaline phosphatase are alike in the two groups (*26*).

There is a certain amount of skepticism as to whether the lesion being discussed is, indeed, a neoplasm. It has been suggested that it is too well differentiated—too much like liver morphologically—to be a neoplasm. One cannot rule out the possibility of neoplasm on the basis of a high degree of differentiation alone. For example, in human material certain metastasizing and at the same time highly differentiated lesions of the thyroid gland and liver should be regarded as tumors. Furthermore, the lesion is very similar to the induced hepatomas in the mouse, and the Golgi apparatus of the cells is indistinguishable from that in induced tumors and quite different from that seen in mere hepatic regeneration. That the lesion is a tumor seems reasonable to accept. Whether it is malignant or benign is a question that awaits further clarification.

Hemangio-endothelioma of the liver occurs occasionally, and we have followed one in C3H mice by continuous transplantation (*32*). The primary tumor was solitary, bloody, reddish-purple, and somewhat cystic. The histologic features of this tumor are interesting, and they also indicate how unreliable histologic criteria

may be when one attempts to prognosticate the behavior of a hemangio-endothelioma. The primary tumor was a well-differentiated angioma with capillary and cavernous blood-filled spaces (fig. 1, *H*). Its general appearance was not unlike the angioma of the liver so often seen in human subjects and was considered histologically benign. It has been readily transplantable and is now in the eighth generation. The microscopic appearance of the transplants very often suggests malignancy, with many solid areas intermingled among definite vascular structures. In two mice bearing first-generation transplants, there were numerous metastases in the liver with histologic pictures indistinguishable from the primary tumor (fig. 1, *I*). A histologically benign tumor may give rise to actively growing transplants, and metastases from such transplants may appear benign on microscopic examination. Examples have been reported in man which have some resemblance to this case in the mouse and which we have reviewed. The patients each appeared with a histologically benign hemangioma which eventually gave rise to metastases, often widespread. The microscopic appearance of the metastases varied from those suggesting a benign tumor to those obviously malignant (*33–35*).

In the transplants of our tumor the solid areas composed of spindle cells brought up for differential diagnosis the possibilities of solid endothelioma, fibrosarcoma, and leiomyosarcoma (*32*). With Mallory's phosphotungstic acid-haematoxylin stain, the cells showed some fibrils which were few in number, short, and extremely delicate. They were more delicate and much shorter than either fibroglia or myoglia fibrils. Furthermore, with the reticulum stain the solid areas differed from either fibrosarcoma or leimyosarcoma, the individual cells of which tumors are usually encircled by reticulum. In our tumor the reticulum was concentrated about the periphery of solid areas (fig. 1, *J*). There was very little reticulum in relation to the individual tumor cells, and what was there was laid down haphazardly. It may be added that in the vascular areas of the tumor the reticulum encircled the vessels in a basement-membranelike fashion (fig. 1, *K*). We interpreted the solid areas as being of endothelial origin on the basis of the evidence with the special stains mentioned, together with the fact that these areas showed transitions through blood-filled clefts to actual vessel formation.

PULMONARY TUMORS

In 1896 during the course of some experiments on bacterial infections Livingood (*36*) discovered five spontaneous tumors in mice, one of which was a spontaneous lung tumor, apparently the first to be reported in the literature. This tumor was found in an albino mouse in which no other growths were noted, and Livingood believed it to be an adenocarcinoma arising within a bronchus. During the period from 1905 to 1914 a large number of cases were reported and described in detail by Haaland (*5*), Tyzzer (*37*), Murray (*7*), Jobling (*38*), and Slye, Holmes, and Wells (*39*). All these tumors were believed to be spontaneous in origin and primary in the lung, although a number of them occurred in mice bearing tumors in other locations, particularly the breast. They were variously diagnosed as adenoma, papillary cystadenoma, or adenocarcinoma. Tyzzer, and Wells, Slye, and Holmes observed growths which were thought to be epidermoid carcinomas. With the exception of this latter type, a reading of the descriptions and reference to the available illustrations strongly suggest the essential histologic similarity of most of them. As to their point of origin, there was no absolute agreement among the various

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authors, although a great many were thought to arise from alveolar epithelium. Tyzzer noted the resemblance of the tumor cells to bronchial epithelium but on the other hand noted in one instance that the tumor was small and completely independent of the bronchi. By the use of serial sections Jobling traced the origin of one growth to papillary outgrowths from the wall of a bronchiole. The largest series in the early literature was studied by Slye, Holmes, and Wells. They concluded that the tumors might arise from either the alveolar or the bronchial epithelium but that it was extremely difficult to decide this point on the character of the tumor cells. However, they believed that the papillary type of tumor arose more frequently from the alveolar epithelium.

Interest in these tumors apparently waned until 1925 when Murphy and Sturm (*40*) induced pulmonary tumors in mice by the repeated cutaneous application of a coal-tar distillate. They gave no detailed report of the histologic character of the tumors but illustrated a growth which was apparently identical with the type described by the earlier workers. Their work was soon repeated by a number of investigators, most of whom simply referred to the induced tumors as pulmonary adenomas or adenocarcinomas. With the establishment of the highly inbred A strain of albino mice by Strong (*41*) in 1921, a suitable test animal for the study of primary lung tumors became available. Bittner (*42*) reported on the normal spontaneous incidence of lung tumors in this strain, and Andervont (*43*) showed that practically all young mice of this strain will develop multiple lung tumors within 2 months following subcutaneous injection of suitable quantities of 1,2,5,6-dibenzanthracene.

We have had the opportunity in recent years to study large numbers of these tumors in several strains of inbred mice, particularly the strain A from the Roscoe B. Jackson Memorial Laboratory. The induced tumors are almost invariably multiple, pearly white in color, and are often stituated close to the pleura so that they may be seen on gross inspection of the surface of the lung. The spontaneous tumors are frequently solitary, but if multiple, usually do not exceed two to four per animal. The tumors appear to grow expansively and are unencapsulated, being surrounded by compressed pulmonary tissue (fig. 2, *A*). Microscopically the spontaneous and induced tumors are similar, most of them presenting a uniform picture of closely packed columns of cuboidal or columnar tumor cells arranged on a rather sparse stroma and with relatively few blood vessels (fig. 2, *B*). The cytoplasm of the cells is generally smooth, slightly acidophilic, and the free borders of the cells are devoid of cilia. The nuclei are single, round or oval, and vary from vesicular to moderately deeply staining types. Mitotic figures, as in the spontaneous variety, are not numerous. Metastases seem to be infrequent although both lungs are frequently riddled with multiple growths.

Wells, Slye, and Holmes (*44*) reported that of 147,132 mice coming to autopsy in the Slye laboratory, where every mouse is allowed to live out its span of life, 2,865 mice (2 percent) had lung tumors. Of these lung tumors 104 (3.6 percent) showed metastasis outside the lungs; all showed metastasis in the mediastinal lymph nodes; many also showed growths along the chest wall and on the diaphragm. Only 10 showed metastases to other organs: 5 to the kidney, 3 to the heart, 1 to the seminal vesicle, and 1 to the skull. Of the 104 cases with metastasis, 33 of the metastatic growths showed more or less the structure of a sarcoma. This was true

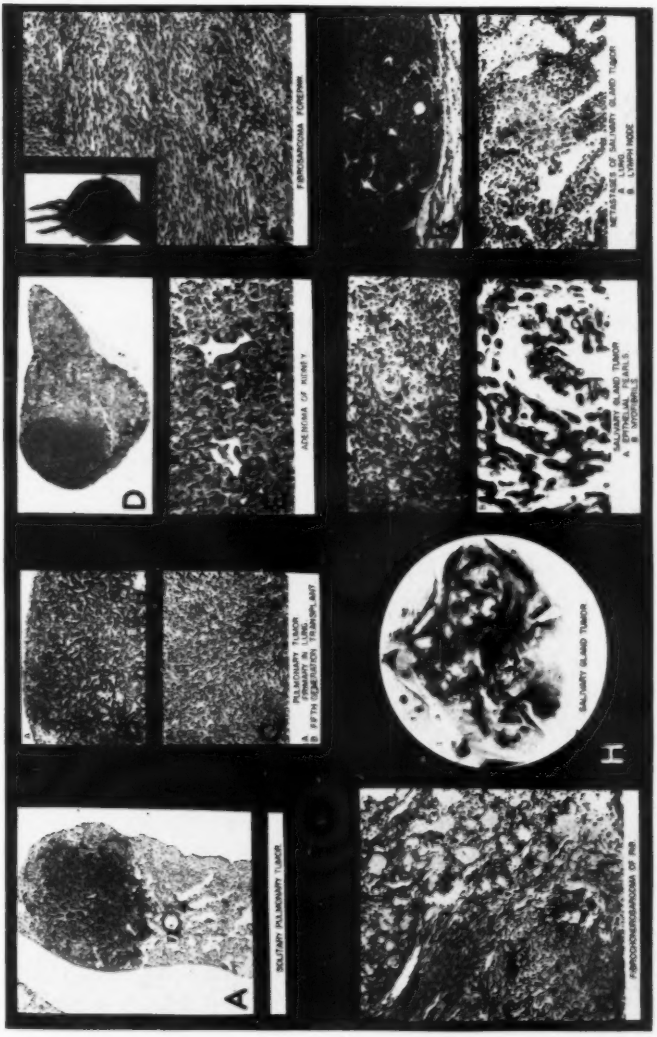
A
SOLITARY PULMONARY TUMOR
B
PULMONARY TUMOR
A PRIMARY IN LUNG
B FIFTH GENERATION TRANSPLANT
D
ADENOMA OF KIDNEY
FIBROSARCOMA PERIORBITAL
FIBROSCHWANNOMA OF RIB
H
SALIVARY GLAND TUMOR
SALIVARY GLAND TUMOR
A EPITHELIAL PEARLS
B MYOMA
METASTASES OF SALIVARY GLAND TUMOR
A LUNG
B LYMPH NODE

FIGURE 2.

even when no sarcomatous elements were identified in the primary growth. Although in a number of primary lung tumors this sarcomatous structure was apparent, no primary sarcomas of the lung without epithelial elements were observed in these mice.

Shimkin (*45*) tested seven strains of mice for their susceptibility to induction of primary pulmonary tumors by the intravenous injection of 20-methylcholanthrene. Strain A was the most susceptible, strain C was next in susceptibility; strains Y, I, and C3H were of medium susceptibility, and strains C57 Black and L were relatively resistant. The relative susceptibility to induction of primary pulmonary tumors was parallel to the susceptibility to spontaneous development of pulmonary tumors, i. e., strains of mice which showed the greatest incidence of induced pulmonary tumors also showed the greatest incidence of spontaneous tumors of the lungs. The pulmonary tumors induced in mice were adenomatous and almost always identical in appearance. There was no morphologic difference (1) between spontaneous and induced tumors, (2) between tumors induced in the seven strains of mice, (3) between tumors produced by methylcholanthrene and those produced by 1,2,5,6-dibenzanthracene, (4) between tumors induced by different routes of injection (subcutaneous, intravenous, intratracheal), or (5) between tumors induced by the carcinogen in different states (in lard, in horse serum, as a cholesterol pellet, or adsorbed on charcoal).

The question of the origin of these tumors in the alveolar walls was discussed by Grady and Stewart (*46*). Young strain A mice received an injection of 1,2,5,6-dibenzanthracene or methylcholanthrene subcutaneously. All animals developing subcutaneous tumors were discarded from the study. The remaining mice were killed daily except Sundays and holidays, beginning on the day following injection, and the experiment was terminated in the eleventh week. Complete stained serial sections of the right lower lobe were examined, and this material yielded pictures showing the various changes occurring in the lung during the development of the pulmonary tumors.

Virtually all the primary induced growths arose in the alveoli and not in the bronchi. Only one tumor in the series was noted in intimate contact with a bronchiole, and this was considered to represent simply partial envelopment of the bronchiole by a tumor of alveolar origin. Grady and Stewart also stated that during the genesis of the tumor there was no associated inflammatory reaction.

It has been noted, as stated above, that when pulmonary tumors metastasize they may show a change in morphology from a papillary glandular type of growth, which they usually assume in the lung, to a spindle-cell growth resembling a sarcoma. The same phenomenon occurs when pulmonary tumors are serially transplanted in the subcutaneous tissue of mice of the same strain as those in which the tumor arose (Andervont). Stewart, Grady, and Andervont studied this morphologic change which occurred following serial transplantation of pulmonary tumors induced by carcinogenic hydrocarbons in various strains of mice including strain A and also in transplants of mouse spontaneous pulmonary tumors.

A portion of each growth was preserved for histologic study. In most instances the transplants eventually developed into tumors composed of closely packed, atypical, spindle cells arranged in interlacing bundles with a characteristic sarcomatous architecture (fig. 2, *C*) and with a greatly accelerated rate of growth. Application of special staining methods including the

phosphotungstic acid-haematoxylin stain for fibroglia fibrils, Foot's silver stain for reticulum, and van Gieson's stain for collagen revealed that these spindle-cell lung-tumor transplants were in fact fibrosarcomas.

RENAL TUMORS

Although inflammatory conditions are very prevalent in the kidneys of mice, a few cases of primary renal tumors have been described among the great numbers of other tumors found in mice. Tyzzer (*13*, *37*) found 4 renal growths which he interpreted as hypernephromas among 83 primary tumors in mice. According to Slye, Holmes, and Wells (*47*), the interpretation of hypernephroma was more liberal when Tyzzer's paper was written than at present, and the original illustrations might now be interpreted as papillary adenoma of the kidney, which was Tyzzer's original diagnosis in 2 of his cases. Among 300 mice with spontaneous tumors described by Haaland (*5*), there were but 2 renal tumors. One, a large growth in the kidney of a 22-month-old male mouse, had invaded a vein but produced no metastases; microscopically, the structure was that of an adenocarcinoma. The second tumor, in a mouse of the same sex and age, resembled perfectly in structure the common hypernephroma type of renal tumor observed in man; there was no metastasis, although the lung contained an adenoma. The second tumor was transplanted into 40 mice, of which 24 survived after 4 weeks with entirely negative results. In 33,000 autopsies performed on mice of the Slye stock up until 1921 only 16 cases of unquestionable primary tumor arising in the kidney were observed, supporting the impression that the kidney of the mouse is not among the common sites of primary neoplasm. The tumors were classified histologically as 1 carcinoma, 3 adenomas, 1 hypernephroma, 7 sarcomas, 3 mesotheliomas, and 1 sarcoma in the renal pelvis. In the material studied by Slye, Holmes, and Wells were several cases of sarcoma of the kidney in mice but no instance of a mixed renal tumor of the Wilms type, which is so common a type of renal tumor in man and apparently also in swine.

Primary tumors of the kidney have been observed in two old strain A mice autopsied in this Institute. One of these was in an untreated mouse. The other mouse was an experimental animal which had received an injection of 3, 4-disodium phenanthrene dicarboxylate (*48*). Since repetition of this experiment failed to yield renal tumors, it is thought possible that the lesion may have been spontaneous.

The mouse was a male approximately 17 months of age. There was a sharply circumscribed, soft, brownish, rounded tumor mass 8 mm. in diameter in the left kidney.

Histologic examination of the specimen revealed a sharply circumscribed tumor in the kidney. The renal tissue showed extensive calcific deposits mainly in the tubules, and there were in addition numerous hyalin casts. Many tubules were dilated and lined by flattened epithelium. There were focal inflammatory areas with fibrosis in the interstitial tissue. The glomeruli enmeshed in the inflammatory areas appeared atrophic and fibrosed, but elsewhere they showed little or no change.

The renal tumor was sharply circumscribed though not encapsulated, and compressed the adjacent renal tissue, appearing to grow expansively (fig. 2, *D*). The cells of the tumor were large and contained a single large round or oval hyperchromatic nucleus (fig. 2, *E*). The nuclei were relatively uniform in size, shape, and staining. The cytoplasm was abundant, slightly basophilic, and finely vacuolated.

Approximately one mitotic figure was observed in every four high-power fields. The cells were arranged in solid alveolar-like clumps which occasionally showed a cordlike or acinous arrangement, surrounded by a thin strand of reticulum and collagen, neither of which demonstrated any tendency to penetrate between the tumor cells. Blood vessels were numerous and consisted of irregularly shaped sinusoidal channels lined by a single layer of flattened endothelium. The connective tissue was scanty, and there were no inflammatory cells.

FIBROBLASTIC TUMORS

In an 8-month-old female mouse of the C3H strain a fibrosarcoma involved the right foreleg and paw (fig. 2, *F*). It had evidently originated in the soft tissues and secondarily invaded the cortex of the ulna, partly replacing the medullary cavity. At the time of writing, this tumor is growing well on transplantation. No alkaline phosphatase has been demonstrated in the cells of transplants. This tumor is being reported in greater detail elsehwere (*19*).

A fibrochondrosarcoma of the rib was observed in a male strain A mouse 1 year of age. The tumor was globular, white, and of cartilaginous consistence involving the pleural aspect of the third right rib anteriorly. It measured 0.5 cm. in diameter and protruded into the thoracic cavity without any break in the parietal pleura. No metastases were observed, and so far (after 3 months) no successful transplants have been obtained.

Histologically, the tumor was composed partly of well-differentiated fibroblasts showing mitotic figures rarely, and associated with considerable collagen and reticulum. Mixed with the fibroblastic tissue were many islands of hyalin cartilage (fig. 2, *G*).

In the submaxillary regions of albino mice of strains C and A the salivary glands are occasionally involved by tumors (fig. 2, *H*) which at times reach a diameter of 2 cm. The tumors are moderately firm and gray and usually solid. Occasionally the larger tumors are cystic and contain mucuslike fluid. The tumor cells are relatively large, with cytoplasm that usually is slightly basophilic and prominent, relatively large, vesicular nuclei (fig. 2, *I* and *J*). The cells tend to grow in sheets or masses which in turn are surrounded by delicate collagen or reticulum. Occasionally there is a tendency toward keratinization and pearl formation (fig. 2, *I*). With the phosphotungstic acid-haematoxylin stain many of the cells contain blue fibrils which are usually numerous and delicate suggesting fibroglia fibrils (fig. 2, *J*). Less often the fibrils are less numerous and stouter resembling myoglia fibrils (fig. 2, *J*). The tumor is transplantable and metastasizes (fig. 2, *K* and *L*). While the exact origin of this neoplasm is still uncertain, it has been suggested that it is a myoepithelioma that has arisen from the so-called basket cells of the salivary gland.

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Carcinogenic Effectiveness of Ultraviolet Radiation of Wavelength 2537A

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That depth of penetration of ultraviolet radiation into the skin may be a limiting factor in determining the types of tumors induced by this agent was pointed out in previous papers from this laboratory (*1–2*). Radiation of wavelength 2537A is almost completely absorbed in the epidermis of the albino mouse (*1*) and might be expected *a priori* to produce a higher proportion of epidermal carcinomas than do longer wavelengths, since for equal incident energies a greater proportion of the latter are transmitted by the epidermis and reach the deeper layers of the skin in greater amount. For this reason, and because this wavelength can be easily obtained as practically monochromatic radiation, it was decided to study its carcinogenic action. It was necessary to end our experiments after 8 months because of high mortality among the animals owing to a liver infection; hence the results do not have quantitative significance. They were continued long enough, however, to permit certain conclusions to be drawn with assurance; and since many months are needed to repeat the experiments, it seems wise to make the results available at this time.

METHOD

The radiation was obtained from two low-pressure mercury arcs enclosed in envelopes of high-transmission glass (General Electric Germicidal Lamps). About 95 percent of the carcinogenic radiation emitted by these arcs is in the 2537A resonance line. The lamps are about 90 cm. long, and with the two lamps placed parallel it was possible to arrange 25 animals in individual cages below them so that all received practically the same intensity of radiation. It was thus unnecessary to rotate the animals as in previous experiments (*3*). The energies and intensities were measured with a titanium photocell and integrating counter (*3*). The intensity in the plane of the animals was approximately 1.4×10^4 ergs per square centimeter per second and hence is within the range in which the reciprocity law has been found to hold for tumor production (*4*, *5*).

Four groups of 25 animals were employed, all male strain A albino mice. The group exposed to the highest dosage received 36×10^7 ergs per square centimeter per week given in equal doses on 5 days per week. The period of exposure required for this dosage was approximately 1½ hours. Two of the groups received, respectively, one-half and one-quarter of this dose. In exposing the fourth group, which served as a control, a Corex D filter was interposed between the lamps and the animals. This filter removes the 2537A line but transmits small amounts of longer carcinogenic wavelengths which are emitted by the source. Thus any effects produced in this control group must be attributed to these longer wavelengths and not to wavelength 2537A. Actually no consistent differences between the controls and unexposed animals were detected.

For histopathologic studies the ears were subjected to the technique previously employed (*2*).

RESULTS

The histopathologic changes observed on the ears of the mice subjected to wavelength 2537A were characterized by hyperkeratosis and acanthosis. The epithelial hyperplasia was both local and widespread. Parakeratosis was infrequent, and benign dyskeratosis was rarely identified. Some proliferation of connective tissue elements, edema, and infiltration of inflammatory cells were observed in the dermis. In general these morphologic alterations varied in degree with the dose of radiation to which the group was subjected, those receiving the lowest dosage showing only minimal changes. There was wide individual variation; a mouse of one group sometimes displayed changes which could not be distinguished from those shown by an animal of another group. The ears of the controls appeared essentially normal showing that the changes observed in the other groups were undoubtedly ascribable to wavelength 2537A. In figure 1, *A* and *B*, the ear of a control is compared with that of an animal receiving the highest dosage of wavelength 2537A.

Tumors were observed only in the group that received the highest dosage. At 227 days one animal showed a tumor of a size corresponding to the established criterion for selection used in previous studies (*2*). This proved to be a fibrosarcoma. All the surviving animals in this group were killed at the end of 241 days. Among these animals and those which had died and had been examined previously, one early fibrosarcoma (fig. 2, *A* and *B*) and a small, early, squamous carcinoma were found. In addition three lesions of the epidermis were observed which might be regarded as squamous carcinoma *in situ*. They are of interest because they appear to have gone beyond the stage of focal hyperplasia, yet one hestitates to classify them as definitely malignant. One of the lesions is illustrated in figure 2, *C* and *D*.

Rusch, Kline, and Baumann (*6*) report that they were unable to induce tumors with wavelength 2537A, but the dosage they employed seems to have been lower than that which produced the above-described tumors, and their experiment was terminated somewhat earlier than ours.

COMPARISON WITH EFFECTS OF OTHER WAVELENGTHS

In all the previous studies reported from this laboratory a type of intermediate pressure mercury arc was employed as the source of radiation (*3*). The carcinogenic radiation of wavelengths shorter than 3200A from this source incident on the surface of the skin is about 12 percent wavelength 2537A, but about 57 percent is of wavelengths 2967A, 3022A, and 3130A. As will be shown later, the radiation incident on the viable cells themselves is that which should be considered in comparing effects of different wavelengths, and this is composed predominantly of the last three wavelengths. This is indicated in table 1, where estimates of the percents of total energy just beneath the epidermis are presented. These are calculated from measured epidermal transmissions (*1*). Since most of the absorption by the epidermis must be due to the dead stratum corneum, these values are reasonably good approximations of the relative energies reaching the viable cells. Thus, for such rough comparisons as are required for the following discussion, the radiation from the intermediate pressure mercury arc may be considered to be approximately of wavelength 3000A.

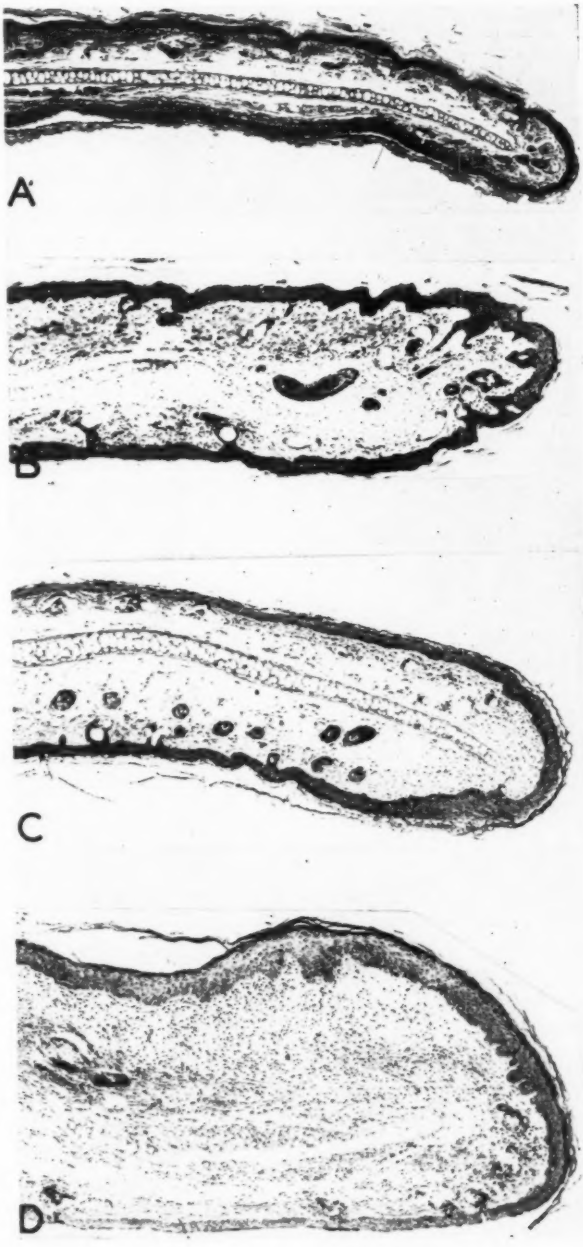


FIGURE 1.—Sections of ears of mice. *A*, Control; *B*, Ear exposed to wavelength 2537A, 36 x 10^7 ergs per square centimeter per week; *C*, Ear exposed to 3.6 x 10^7 ergs per square centimeter per week of all radiation of wavelength 3130A and shorter, from an intermediate pressure mercury arc; *D*, section of ear exposed to same type of radiation as *C*, 16.5 x 10^7 ergs per square centimeter per week. All dosages given in five equal exposures per week, and all sections stained with haematoxylin and eosin. × 55.

TABLE 1.—*Estimation of total energy incident on the surface and beneath the epidermis of the ears of mice*

Wavelength	Energy of carcinogenic wavelengths (shorter than 3200A)	
	Incident on surface	Incident beneath the epidermis
	Percent of total	*Percent of total* [1]
3130A	32	58
3022A	16	24
2967A	9	10
2925A	1	1
2894A	3	2
2804A	6	1
2752A	2	
2699A	2	
2652A	11	2
2576A	1	
2537A	12	2
2483A	3	
2399A	1	

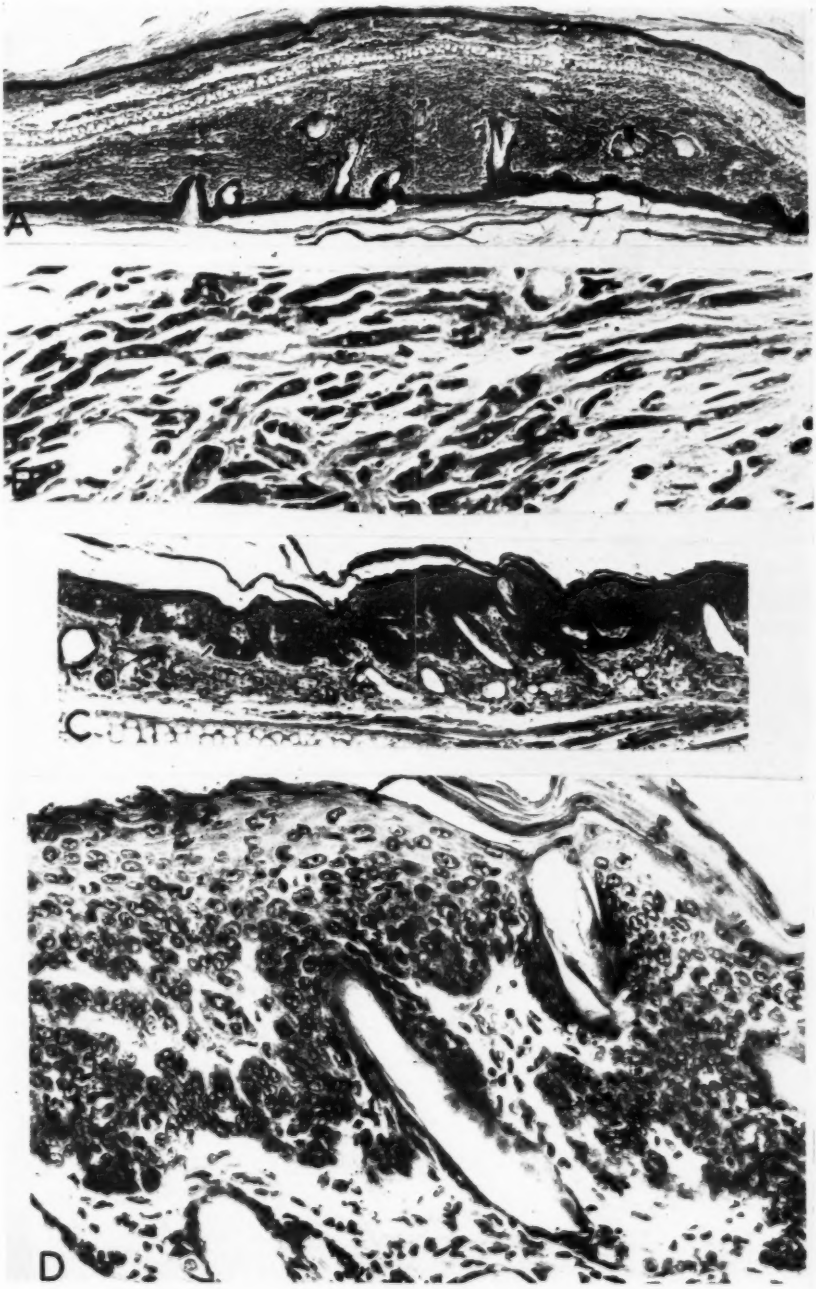
[1] Estimated from measurements of transmission of separated epidermis (*1*).

In figure 1, *B* and *C* represent the ears of mice which received, respectively, 36 x 10^7 ergs per square centimeter per week of wavelength 2537A and 3.6 x 10^7 ergs per square centimeter per week of radiation from the intermediate pressure mercury arc. Obviously the extent of the changes, as indicated by comparison with the control *A*, are quite comparable in the two cases, yet *B* received 10 times as much total carcinogenic radiation on the surface of the skin as did *C*. When the amounts of radiation reaching the viable cells is considered, the discrepancy in dosage is not nearly so great, however, for only about 2 percent of wavelength 2537A penetrates to this layer as compared with about 15 percent of wavelength 3000A which may be considered characteristic of the intermediate pressure arc (*1*). Thus, the dosages are roughly equivalent, and no great difference, either quantitative or qualitative, can be attributed to the effect of the different wavelengths when incident upon the viable cells. Figure 1, *D*, represents a surface dosage of 16.5 x 10^7 ergs per square centimeter per week, or approximately one-half the surface dosage represented by *B*, but the changes are much more extensive. Obviously, the 2537A line is relatively ineffective in terms of surface dosage, although it has approximately the same effectiveness when considered in terms of energy reaching the viable cells.

The same relationship seems to apply as regards tumor induction, although no accurate comparison can be made because of the early termination of the experiment. The first tumor induced by wavelength 2537A of the same dosage represented in figure 1, *B*, was 227 days, which falls among the induction times of first tumor in five experiments in which the animals received the same dose of intermediate pressure arc radiation represented by figure 1, *C*. Thus we may conclude that the effectiveness of wavelength 2537A in tumor induction is roughly the same as that of wavelengths near 3000A when considered in terms of energy reaching the viable cells, although the wavelength is much less effective when considered in terms of energy striking the surface of the skin.

The fact that a number of epidermal lesions were found which might be expected to develop into carcinomas suggests that, other things being equal, wavelength 2537A may induce a higher percent of carcinomas than do longer wavelengths. However, this is not definitely established by these experiments; and recent studies (*7*) indicate that while penetration of the radiation must always be a limiting factor, the interval between exposures to ultraviolet radiation may be more important in determining the type of tumor induced.

FIGURE 2.—*A*, Early fibrosarcoma. × 55; *B*, Same neoplasm. × 450; *C*, Proliferating epidermis suggesting so-called carcinoma in *situ*. × 80; *D*, Same neoplasm. × 335. Haematoxylin and eosin stain used for all.


A
B
C
D

SUMMARY AND CONCLUSIONS

Considered in terms of surface dosage, wavelength 2537A is much less effective than longer wavelengths of the carcinogenic spectrum in inducing tumors and in producing other tissue changes. This is best explained by the fact that wavelength 2537A is absorbed principally in the stratum corneum before it reaches the viable cells. Thus it appears evident that photochemical changes taking place in the stratum corneum play no part in carcinogenesis and that the locus of carcinogenic action of ultraviolet radiation is the living cell itself.

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Influence of Limited Application of Methylcholanthrene Upon Epidermal Iron and Ascorbic Acid[1]

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In the group of investigations of epidermal methylcholanthrene carcinogenesis which are being carried out at this hospital, a correlation of the chemical, histologic, and physical properties of the cells is desired for a better understanding of the problem. In this paper, studies on total iron and ascorbic acid content of the epidermis are reported.

The difficulty of obtaining adequate material for studying progressive changes of normal cells to malignant ones has often retarded a systematic study of carcinogenesis. However, for the epidermis, the heat method of separation from the dermis, as devised by Baumberger, Suntzeff, and Cowdry (*1*), now permits a study of the chemistry of epidermal carcinogenesis.

Another difficulty in comparing the chemical constituents of normal and cancerous tissue is that of an adequate base. This was especially true in the present work in comparing normal epidermis, a comparatively homogeneous tissue, with late hyperplasias, papillomas, and carcinomas, which are often quite heterogeneous, owing particularly to extensive keratinization. In our investigation, the basis of reference was nucleoprotein phosphorus, which had been shown to be adequate by Berenblum, Chain, and Heatley (*2*). It is especially valuable as results may then be expressed in terms of viable cells. This can be expected to give a much better indication of the events in carcinogenesis.

EXPERIMENTAL PROCEDURE

New Buffalo mice of both sexes 3 to 4 months old were used, as there was no apparent sex difference. The entire area of the back of each mouse was shaved with the aid of distilled water only, and after a 2-or-3-day rest the mice were given treatment as follows: The benzene-treated control mice were painted with reagent-grade benzene, and the methylcholanthrene-treated mice with a 0.6 percent (weight/volume) solution of that carcinogen in the same solvent. The epilated area was painted by making one broad stroke down the back with a No. 5 camel's-hair brush. The normal controls were used for analysis 10 days after shaving, and all groups were dry shaved if necessary to remove any regrown hair prior to separating the epidermis. The epidermis was detached at 50° C. by blunt dissection by using the technique mentioned above.

For the determination of total iron and nucleoprotein phosphorus, the fresh samples were weighed to the nearest 0.1 mg. as they were to be divided into two parts. The larger portion was treated as follows for the determination of iron: Each sample was dry ashed at approximately 450° C. in a muffle furnace until all organic matter was destroyed. The ash was dissolved in 1 cc. of N/10 hydrochloric acid, and one drop of concentrated nitric

[1] *Aided in part by a grant from the National Cancer Institute.*

acid was then added to insure complete oxidation of the iron to the ferric state. The solutions of the ash, while still in the original silica crucibles, were brought to dryness on a steam bath, and the salts were dissolved out with 1 cc. (recalibrated pipette) of 1 M potassium oxalate, which Stackelburg, Freihold, and Lingane (*3, p. 276*) have proved to be a good supporting electrolyte when ferric iron is to be determined polarographically. Immediately afterward, the iron analyses were made at 25° with a Heyrovsky polarograph, model XI.

The smaller portion of the sample of epidermis was treated for the determination of nucleoprotein phosphorus. The method of extraction of the tissue was that of Berenblum et al. (*2*). The residue, free of fat and acid-soluble phosphorus, was digested with mixed nitric and perchloric acids, and the resulting orthophosphate was determined by the sensitive method of Truog and Meyer (*4*). A spectrophotometer was employed for determining the intensity of the blue color produced by the reduction of ammonium phosphomolybdate by stannous chloride, all measurements being made at 710 mμ and at 5 to 6 minutes after the addition of the reductant. Timing was necessary as there was a slow change in the intensity of the color.

The ascorbic acid content of the tissues was determined by an improved photometric method [2] and calculated both on a weight basis and in relation to the nucleoprotein phosphorus content.

RESULTS

Total Iron

A comparison of the analyses for total iron of normal controls, benzene-treated controls, and methylcholanthrene-treated

[2] *Carruthers, C.: Indust. & Engin. Chem., Analyt. Ed.; (In press).*

Table 1.—*Iron-nucleoprotein phosphours ratio of mouse epidermis*

Number of mice	Number of paintings	Time after first treatment to killing of mice	Fe per 100 mg. tissue	N.P. P. per 100 mg. tissue	Fe/N.P. P.
		Days	*Micrograms*	*Milligrams*	
NORMAL, UNTREATED MICE					
10			7.1	0.095	75
9			5.4	.117	46
8			6.9	.126	55
7			9.2	.119	77
8			6.7	.125	53
9			5.1	.141	36
12			6.4	.132	48
12			6.8	.126	54
4			7.7	.125	62
11			6.9	.135	51
8			4.6	.136	34
10			4.3	.121	36
108	(Total) Average		6.4	.125	52
BENZENE-TREATED MICE					
9	3	10	6.4	0.142	45
9	3	10	6.6	.119	55
8	3	10	9.1	.095	96
9	3	10	8.0	.094	85
8	5	14	11.7	.114	102
10	9	20	9.5	.128	74
10	10	24	5.2	.133	39
63	(Total) Average		8.1	.118	71
METHYLCHOLANTHRENE-TREATED MICE					
8	1	10	3.7	0.127	29
5	1	10	3.2	.116	28
4	1	10	3.3	.119	28
8	1	10	3.1	.131	24
25	(Total) Average		3.3	.123	27
8	2	10	2.1	.135	16
6	2	10	2.9	.152	19
7	2	10	4.5	.142	32
21	(Total) Average		3.2	.143	22
8	3	10	2.2	.092	24
5	3	10	1.8	.105	17
7	3	10	3.1	.108	29
20	(Total) Average		2.4	.102	23
4	6	15	3.0	.105	29
5	6	15	2.4	.080	30
8	7	14	2.4	.123	20
7	7	21	1.9	.132	14
5	8	20	2.7	.094	29
4	9	26	2.3	.090	26
33	(Total) Average		2.5	.104	25
4	13	30	1.5	.120	12
5	13	30	1.5	.118	13
4	18	40	1.5	.086	17
4	33	70	1.9	.102	18
3	33	70	1.4	.122	11
20	(Total) Average		1.6	.110	14

mice is shown in table 1. The results are expressed in terms of micrograms of iron (Fe) per 100 mg. of tissue, of milligrams of nucleoprotein phosphorus per 100 mg. of tissue (denoted by N. P. P.), and also by the significant ratio Fe/N. P. P. Since different samples of the separated epidermis varied in their moisture content, an unavoidable feature of the heat separation technique, expression of the constituents on a weight basis was not practical. However, the ratio Fe/N. P. P. compensates for hydration changes as separate portions of the tissues were accurately weighed when divided. The epidermis of the normal untreated mice contained an average of 6.4 micrograms of iron per 100 mg. of tissue, and an Fe/N. P. P. ratio of 53. There was a significant increase in the iron of the benzene-treated controls, an average iron content of 8.1 micrograms per 100 mg. of tissue and an Fe/N. P. P. ratio of 71 . On the other hand, one application of methylcholanthrene, despite the rise owing to benzene, was sufficient to reduce the iron content to an average of 3.3 micrograms per 100 mg. of tissue and to lower the ratio to 27 when the mice were killed 10 days after painting. Two to nine applications produced similar changes when the animals were killed 10 to 26 days after the first treatment with the carcinogen. Repeated applications (30 to 70 days) caused a further decrease in the total iron content and in the Fe/N. P. P. ratio.

Ascorbic Acid

The ascorbic acid content of the epidermis following treatment with methylcholanthrene is shown in table 2. Samples were taken from 1 to 12 days after one to three applications of the carcinogen. The ascorbic acid content calculated on a weight basis of the original tissue is significantly less in carcinomas than in the epidermis of normal mice, benzene-treated controls, or methylcholanthrene-treated mice. However, the ratio of ascorbic acid to nucleoprotein phosphorus was nearly the same for all these groups. Here the importance of an adequate base is exemplified, a lack of which may well explain some of the alleged quantitative differences between normal and cancerous cells reported in the literature where the results were usually expressed on a weight basis.

Table 2.—*Ascorbic acid in epidermal carcinogenesis*

Group	Analyses	Total mice	Paintings	Time after first treatment to killing of mice	Ascorbic acid per gram of tissue	Nucleoprotein phosphorus per gram of tissue	Ascorbic acid/ N. P. P.
	Number	*Number*	*Number*	*Days*	*Milligrams*	*Milligrams*	
Normal, untreated mice	5	16	0	(1)	0.239	1.24	0.193
Benzene-treated mice	5	16	1	10	.206	1.19	.173
Methylcholanthrene-treated mice	4	11	1	10	.224	1.34	.167
	6	19	1-3	1-12	.222	1.30	.171
Carcinomas	5	5			.138	.75	.184

[1] Killed 10 days after first shaving.

DISCUSSION

The process of epidermal carcinogenesis no longer appears to require a continuous application of a carcinogen (*5*). A single treatment may yield a massive epithelial hyperplasia in a fraction of the mice and even a carcinoma (*6*, *7*). Mider and Morton (*8*) induced a small percentage of

carcinomas in C57 Brown mice, and in this hospital Cramer and Stowell [3] obtained an even higher percentage in mice of the Swiss strain. That there is an early reaction to the carcinogen is also demonstrated by the work of Cooper and Reller (*9*) of this hospital, who found an increase in the mitotic count in epidermis of the ears of mice 48 hours after a single application of methylcholanthrene.

However, no quantitative chemical data have been presented to show that the response to methylcholanthrene is immediate. The significant lowering of the total iron in this study and the marked decrease in the total lipid found by Wicks and Suntzeff (*10*) demonstrate for the first time that one application of methylcholanthrene can elicit an early change in chemical composition as well. These observations also show that the reaction to a carcinogen cannot be ascribed to any single change but probably to many occurring simultaneously.

There is no significant change evident in the ascorbic acid content of the epidermis undergoing carcinogenesis, but this study has demonstrated the need of an adequate base to prevent wrong conclusions.

[3] *Cancer Research, 2: (In press).*

SUMMARY

A single application of methylcholanthrene reduced the total iron content of the epidermis of mice within a few days to approximately 50 percent of the normal content. Multiple applications of the carcinogen applied on alternate days induced a further lowering. Ascorbic acid, on the other hand, was not significantly altered during the course of epidermal carcinogenesis.

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Reduction of Total Lipid-Protein Nitrogen Ratio of Mouse Epidermis by a Single Application of Methylcholanthrene[1]

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During an investigation of possible alterations in the lipids of the epidermis of mice treated topically with a benzene solution of methylcholanthrene, one change stood out conspicuously and consistently. This was the marked and sudden reduction in the total epidermal lipid material. Five days after even a single application of the carcinogen, the total fatty substance of the epidermis fell to less than 40 percent of that at the normal level. This effect was due to the carcinogen alone and not to the solvent, for values of the benzene-treated controls agreed well with those of the untreated skins.

EXPERIMENTAL PROCEDURE

With the exceptions shown in table 1, New Buffalo strain mice between 3½ and 5 months old were used. Both males and females were employed as no sex difference was evident. All the animals were shaved over the entire area of their backs, only distilled water being used. They were then allowed to rest for 2 to 3 days before any other treatment. The treated mice were painted, with a No. 5 camel's-hair brush, over their backs either with benzene alone (benzene-treated controls), or with a 0.6-percent (weight/volume) solution of 20-methylcholanthrene in benzene. Of the methylcholanthrene-treated animals, most of the groups were killed at 2, 3, 5, or 10 days after a single application. Some animals, which received more than 1 treatment, were painted on alternate days 2 to 7 times and were killed 10 to 15 days following the initial application. The benzene-treated controls received 3 to 17 treatments, also on alternate days, and were killed 10 to 35 days after the first treatment (table 1). The untreated controls were shaved 10 days prior to the removal of the skins. Before any of the animals were killed, they were again dry shaved where necessary. They were then chloroformed, and a sheet of skin was removed which was well within the borders of the treated area. The epidermis was separated by the heat and blunt dissection method of Baumberger, Suntzeff, and Cowdry (*1*) developed at this hospital. Pooled pieces of epidermis from 4 to 14 animals were used for extraction.

The histologic picture of the epidermis of the untreated control group showed two layers of undifferentiated epithelial cells above which was a thin layer of keratin (fig. 1), while that of the benzene-treated controls was different. The epidermis of mice which had been painted with benzene five times and killed 10 days after the first application displayed the beginning of differentiation of the epidermis. This consisted of four to five layers of cells distal to which there was a thin layer of keratin. A few mitotic figures were present in the basal layer of the epidermis (fig.

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TABLE 1.—*Lipid-protein N ratio of mouse epidermis*

Group of mice	Number	Treatments	Time after first treatment until killing of mice	Total lipid	Protein N	Lipid/protein N ratio
		Number	*Days*	*Milligrams*	*Milligrams*	
Normal, untreated	[1] 8			26.8	5.3	5.06
	[2] 9			55.8	11.2	4.98
	[1] 14			61.4	12.0	5.10
	12			49.3	10.4	4.74
	6			34.9	5.2	6.71
	8			50.7	10.3	4.92
Total or average	57					5.25
Benzene-treated	12	3	10	48.7	8.7	5.60
	7	5	10	51.2	9.4	5.45
	9	5	10	38.9	6.7	5.81
	8	6	15	55.6	13.2	4.21
	11	7	14	57.6	11.2	5.14
	10	17	35	45.8	9.5	4.82
Total or average	57					5.17
Methylcholanthrene-treated	5	1	2	46.6	13.8	3.38
	5	1	2	51.9	14.8	3.51
	5	1	2	48.2	10.9	4.42
	5	1	2	40.7	7.0	5.81
Total or average	20					4.28
	5	1	3	39.9	10.8	3.70
	6	1	3	41.6	14.8	2.81
	5	1	3	50.6	11.2	4.52
	5	1	3	50.7	13.2	3.84
Total or average	21					3.72
	8	1	5	60.1	30.5	1.97
	5	1	5	38.6	22.2	1.74
	4	1	5	45.9	20.2	2.27
Total or average	17					1.99
	8	1	10	43.8	20.6	2.13
	8	1	10	49.0	15.1	3.24
	10	1	10	43.7	19.8	2.21
Total or average	26					2.53
	5	2	10	40.8	16.2	2.52
	8	2	10	44.4	31.1	1.43
	7	3	10	49.5	37.1	1.33
	9	3	10	45.6	17.6	2.59
	5	4	10	43.4	21.5	2.02
	11	6	14	54.0	22.9	2.36
	4	7	15	40.0	16.2	2.47
Total or average	49					2.10

[1] Mixed stock. [2] Swiss strain.

2). The skins which were removed from mice killed 5 days after a single application of methylcholanthrene exhibited an irregularly differentiated epithelium consisting of six to seven layers of cells. There was a slight increase in the amount of keratin on the surface of the epidermis over that found in either control group. A number of mitoses were in the basal layer (fig. 3). Central sections of skin taken for histologic examination were from 60 to 70 percent of the tissues used for analysis.

The samples of epidermis were examined closely for any hair, then cut into small pieces with shears, and transferred to 50-cc. flasks which were fitted with West reflux condensers. Extraction of the lipids was as follows: 1½ hours; 1 hour; three-fourths of an hour, directly in boiling 3:1 alcohol-ether mixture with complete change of solvent each time. The tissue residues

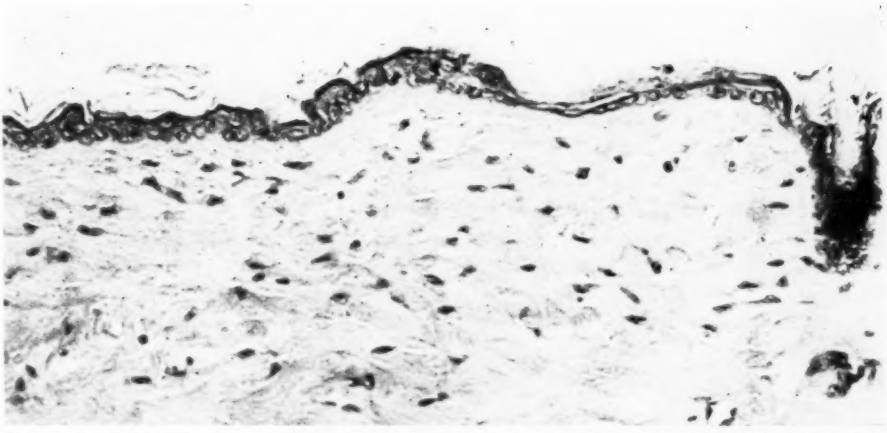

FIGURE 1.—Epidermis of an untreated control mouse consisting of two layers of undifferentiated epithelial cells with thin layer of keratin on the surface. × 270.

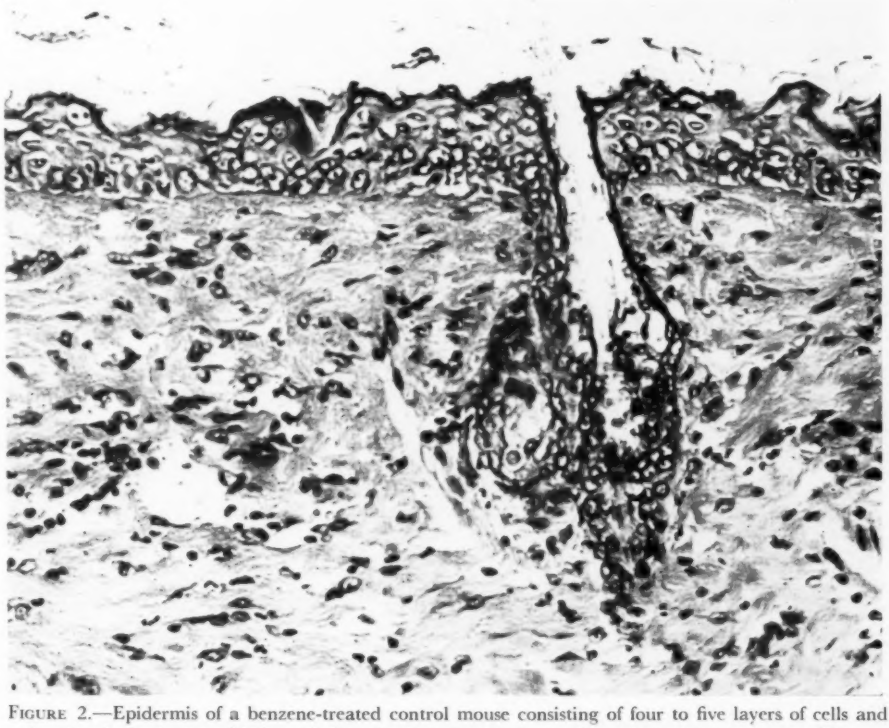

FIGURE 2.—Epidermis of a benzene-treated control mouse consisting of four to five layers of cells and showing the beginning of differentiation of cells and a thin layer of keratin on the surface. × 270.

were caught on a filter (Whatman No. 1 paper) and washed repeatedly with boiling ethyl ether. This manner of extraction was found to be efficient by digesting the tissue residue with 10 percent sodium hydroxide, acidifying with hydrochloric acid, extracting with petroleum ether, and evaporating to examine for any lipid residue. The pooled extracts and washings were carefully evaporated to final dryness on the steam bath with the aid of a stream of carbon dioxide. The ethyl ether was Mallinckrodt No. 0844 for fat extraction; the alcohol (95 percent U. S. P.) was redistilled.

The alcohol-ether residues definitely contained some nonlipid material and were therefore reextracted repeatedly with petroleum ether (Mallinckrodt No. 4980, 30°–60°) and filtered through Munktell 0A paper into weighed 50-cc. glass-stoppered Erlenmeyer flasks. The petroleum ether extracts were carefully brought to dryness on the steam bath, a gentle stream of carbon dioxide was used to finish the process, and the flasks were reweighed. The lipid residues averaged about 40 mg., a quantity which could be determined with accuracy.

As a base for the amount of total lipid, the original weight of the separated epidermis could not be used since the water content of the samples as received was by no means constant. The reference chosen was the protein nitrogen in the lipid-free residue, such a base doubtless being a reliable measure of the amount of tissue involved. The ratio was then expressed as milligrams of total lipid/milligrams of protein nitrogen. Since the samples differed in size and weight as well as in water content, the columns for total lipid and

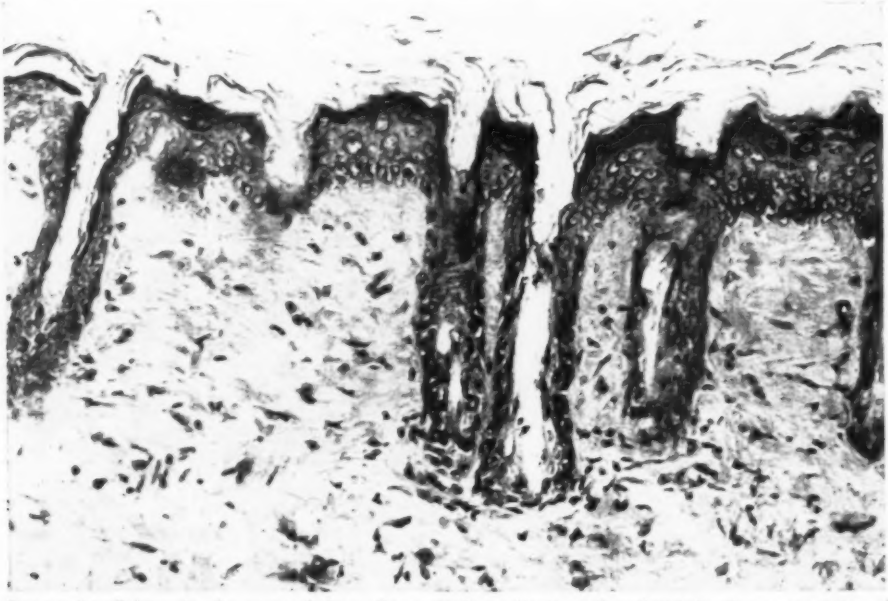

FIGURE 3.—Epidermis of a mouse 5 days after a single application of methylcholanthrene consisting of six to seven layers of differentiated cells with a number of mitoses in the basal layer and with slight increase in the amount of keratin on the surface. × 270.

protein nitrogen in table 1 are given only to show the derivation of the ratio.

For the Kjeldahl digestion, the extracted tissue residues were dried briefly and then digested with concentrated sulphuric acid containing 0.2 percent selenium oxychloride as catalyst. Ignition was completed by the addition of 30 percent hydrogen peroxide. These Kjeldahl digests were diluted to definite volumes with ammonia-free water, and suitable aliquots were taken for direct nesslerization. To use larger aliquots for greater accuracy, the nesslerized solutions were stabilized by the addition of specially purified gum ghatti solution (*2*) so that 2 to 3 mg. nitrogen could be present per 100 cc. of solution.

RESULTS AND DISCUSSION

As shown in table 1, the ratio of total lipid to protein nitrogen in the epidermis was affected little or none by repeated applications of benzene, but even a single treatment with the benzene solution of the carcinogen caused a definite fall in total lipid so measured after only 2 days, a change which was greater at 5 or 10 days. Repeated applications of methylcholanthrene intensified the reduction. Any conclusion as to the significance of the abrupt lowering of this material must be speculation only. Analyses of the individual lipid constituents revealed that of the normal epidermal lipid about 12 percent was phospholipid and cholesterol compounds, the bulk of the remainder presumably being the glyceryl esters of "neutral fat."

There is now little doubt but that rather profound alterations can occur in the skin with but a single application of a carcinogen. Mider and Morton (*3*) obtained papillomas and a few carcinomas after a single treatment of the skins of C57 Brown mice with a dilute solution of methylcholanthrene. Cramer and Stowell (*4*, *5*) of this hospital have obtained carcinomas with but one application of the same hydrocarbon in 40 percent of mice of the Swiss strain.[2] That alterations in the epidermis are initiated very soon after contact with the carcinogen was shown by Cooper and Reller (*6*), who found a definite increase in mitoses of the epidermis of the mouse's ear only 48 hours after a single application of methylcholanthrene. Chemically, Carruthers and Suntzeff (*7*) report that 10 days after a single application of methylcholanthrene there is a marked lowering in the total iron of the mouse epidermis.

Unfortunately, very little work has been done on the lipids of the epidermis under different influences. Kooyman (*8*) found, among other changes, a general decrease in human epidermal lipid during keratinization. Whatever may be the meaning of the fall in epidermal lipid following methylcholanthrene, as presented here, it is strongly suggestive of a disturbance in lipid metabolism. Rusch and Kline (*9*), in abstracting a report of some of their work on the lipids, made this statement:

> Since the carcinogens are soluble in fats and are water-insoluble, these data appear to substantiate the theory that the mechanism of carcinogenesis could be explained as an interference with lipid metabolism.

Be that as it may, this brief report is interesting in that it perhaps represents the first quantitative findings of an early change in the chemical composition of the epidermis in response to a potent carcinogen. It might be mentioned that investigations by one of us (Wicks) on whole skin of treated mice failed to disclose any lipid changes at all. Any alterations seem to occur primarily in the epidermis and are masked by the large amount of unaffected dermis when whole skin is analyzed.

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[2] *Cramer, W., and Stowell, R. E.: Unpublished work.*

SUMMARY

The total lipid material extractable from the epidermis of mice was found to be much lowered a few days after even a single topical application of methylcholanthrene in benzene. Mice treated with benzene alone showed no such reduction. Protein nitrogen was used as a base to represent the amount of tissue involved.

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